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THE RICKETTSIA, *WOLBACHIA PIPIENTIS* (GEN. ET SP.N.) AND ASSOCIATED INCLUSIONS OF THE MOSQUITO, *CULEX PIPIENS*

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(With Plates XIII–XVII, containing Figs. 1–41, and 1 Text-figure)

CONTENTS

PAGE

Introduction 453
Materials and technique 454
I. The rickettsia, *Wolbachia pipientis* (gen. et sp.n.), of *Culex pipiens* . . 455
Occurrence 455
Morphology of *Wolbachia pipientis* 455
Enlarged coccoids and their relation to other forms . . . 457
Histopathology of rickettsia-infected gonads 463
Pathological germ cells 464
Relationship of rickettsia to host 468
Some phases of the problem of classifying the rickettsiae . . . 468
Forms of rickettsiae other than typical rods and coccoids . . 469
The problem of subdividing the rickettsiae 471
The classification of *Wolbachia pipientis* (gen. et sp.n.) . . . 471
II. Cell inclusions (NR bodies) of the gonads of *Culex pipiens*, which may simulate both masses of rickettsiae and the inclusion bodies of certain viruses . 472
Distribution of NR bodies in gonads 474
Pathological follicles 475
Morphology of NR bodies 476
Incidence of NR bodies in different mosquito strains 479
Nature of NR bodies 480
Inoculation and cultivation experiments 480
Summary 481
References 482
Explanation of Plates 483

INTRODUCTION

THE presence of a rickettsia-like micro-organism in the gonads of *Culex pipiens* was reported by Hertig & Wolbach in 1924. They found that this organism possessed the morphology and staining characteristics of rickettsiae in general, that it was always present in the gonads but apparently in no other organ, and that it increased in number with the development of the gonads. Their studies were based on smears only and included no histological details of infected cells or organs. Certain peculiarities of morphology exhibited by this organism and by a number of other non-pathogenic rickettsiae, which are not readily interpreted in terms of ordinary bacterial morphology, led the writer

to undertake further studies on the mosquito rickettsia in the hope of throwing light on the long-debated problem of the nature and classification of the rickettsiae.

It was supposed that this mosquito rickettsia had achieved a relationship to its host approaching that of some of the well-known intracellular symbionts of insects, but it was found during the course of this study that the organism apparently causes degeneration of some of the germ cells, which become the "pathological" cells previously noted by cytologists (Whiting, 1917). In addition to the rickettsiae there were found, as constant features of the gonads, large cell inclusions, termed *NR bodies* on account of their affinity for neutral red, composed usually of granules and rods embedded in a matrix, which superficially resemble masses of rickettsiae on the one hand and the inclusion bodies of certain viruses on the other. The NR bodies were found to be associated with a second pathological condition in which primary follicles of the ovary are occasionally destroyed. The rickettsiae (section I), the NR bodies (section II) and the pathological conditions with which they are respectively associated, form the subjects of this report. Although the rickettsiae and the inclusions are apparently unrelated, in view of their close association in the same organ and the necessity of distinguishing between them, it seemed desirable to report them together.

For reasons discussed later, there is proposed for the rickettsia of *Culex pipiens* the name *Wolbachia pipientis* as a new genus and species of the rickettsiae. The terms rickettsia and rickettsiae are used by the writer in a general sense and refer to the group of minute, Gram-negative, usually intracellular, bacterium-like organisms occurring in arthropods, which includes not only those which have been named as species of the genera *Rickettsia* and *Dermacentroxenus* but also a considerably greater number of unnamed "rickettsia-like micro-organisms" described by Cowdry (1923), Hertig & Wolbach (1924) and others. In this broader sense rickettsia is a common name already somewhat widely current and is comparable in these respects to amoeba, coccidium, etc.

Materials and technique

Material of *Culex pipiens* and other mosquitoes was obtained from the vicinity of Boston during 1933, 1934 and 1935, and included hibernating females taken from cellars from January to March, and larvae, pupae and adults reared from egg-rafts collected from August to November, together with a few adults caught at various times during the season.

Smears were usually fixed in absolute alcohol or in osmic vapour, and stained with Giemsa's stain in dilutions ranging from 1 : 10 to 1 : 40 for intervals ranging from 15 min. to several hours. Material for sectioning was fixed chiefly in Regaud's fluid, Zenker's fluid or Carnoy's alcohol-acetic-chloroform mixture, cleared in chloroform and embedded in paraffin. In some cases the gonads were dissected out before fixation, though there was no difficulty in obtaining

good sections of the whole abdomen or of the entire insect, provided the body wall was cut before fixation. The siphon and anal segment of larvae were cut off; in the case of pupae and adults, legs and wings were cut off and at times incisions in the thorax were also made. All sections were cut at 3μ. The most uniformly satisfactory preparations were those fixed in Regaud and stained from 3 to 24 hours with Giemsa. Differentiation was more satisfactory from the standpoint of the rickettsiae if done in acetone rather than in alcohol. As is characteristic of Giemsa sections, results varied greatly for no obvious reason.

I. THE RICKETTSIA, *WOLBACHIA PIPIENTIS* (GEN. ET SP.N.), OF *CULEX PIPIENS*

Occurrence

Hertig & Wolbach's (1924) description of the rickettsia of *Culex pipiens* was based on twenty-five males and females taken at Boston and Minneapolis and on larvae reared from two egg-rafts. The writer found each of several specimens of this mosquito caught in Peiping, China, in 1924 to be similarly infected. In the present study, in which several hundred individuals have been examined, the invariable occurrence of this rickettsia has been confirmed. The description by Lomen (1914) and Whiting (1917) of the "pathological cells" in the testis of *C. pipiens* permits the assumption that their material was infected with this rickettsia. Schaudinn (1904) recorded the presence of yeasts in the oesophageal diverticula of *C. pipiens* and considered certain minute bodies found in the ovaries as possible "fruiting forms" in the life cycle. There is no basis for determining what these bodies were, but it is quite possible that they were the rickettsiae. The writer knows of no other report of this rickettsia nor of the finding of rickettsiae in the gonads of any other mosquito.

Hertig & Wolbach reported as negative for rickettsiae, *Aedes stimulans*, *A. sollicitans*, *A. impiger*, *Culex pulchriventer* (?), *Uranotaenia* sp., and *Anopheles maculipennis*. The following mosquitoes examined by the writer have also been negative: *Culex territans*, a number of specimens of all ages reared from egg-rafts collected during two seasons; *C. apicalis*, 1 ♀; *Culex* sp., 2 ♂♂; *Aedes aegypti*, several adults and larvae of a laboratory strain kindly provided by Dr A. W. Sellards; *A. vexans*, 1 ♀; *A. triseriatus*, 1 ♀; *A. excrucians*, 2 ♀♀; *A. hirsuteron* (?), 1 ♀; *Anopheles quadrimaculatus*, 2 ♀♀, 3 ♂♂; *A. punctipennis*, 3 ♀♀. While these data afford no conclusive evidence as to the non-occurrence of rickettsiae in other mosquitoes, the examinations were such that an infection comparable with that of *Culex pipiens*, had it been present, would have been revealed.

Morphology of Wolbachia pipientis

Stained smears. The morphology of the rickettsiae is most conveniently studied in smears. Since every germ cell of *Culex pipiens* is infected, smears of the gonads yield large numbers of organisms in every field. This rickettsia

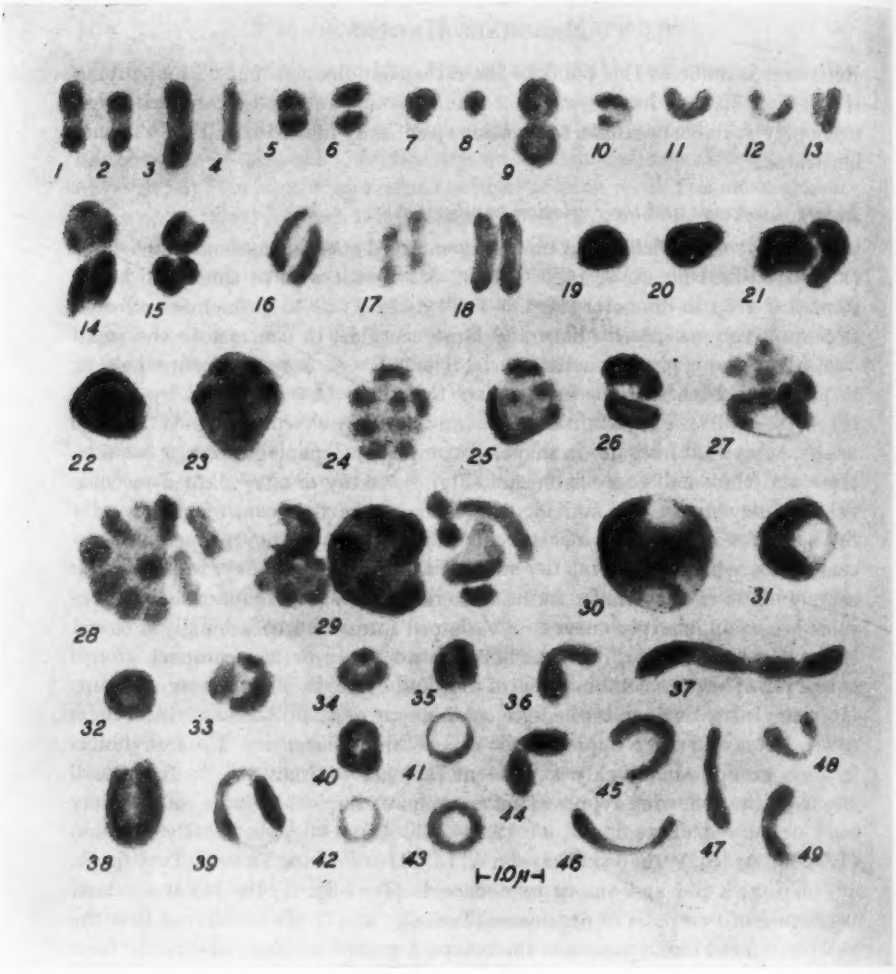
exhibits a degree of pleomorphism unusual even in this characteristically pleomorphic group of micro-organisms. While in the case of most rickettsiae certain forms tend to predominate and whole fields may be composed of more or less uniform rods or coccoid bodies, this rickettsia exhibits almost always a mixture of forms. It is seldom that an infected cell or even a cell fragment contains but a single form. Indeed, it is difficult to designate any one form as typical. However, the almost invariable presence of curved or sharply bent rods, and of large, irregular coccoids, and the apparent aggregation of several organisms into tiny masses of uniform size, give a characteristic appearance to smears of this organism. To avoid the taxonomic connotation of *coccus*, the term *coccoid* is used for the various forms which are more or less spherical in contrast to those which are rod-shaped. It may be stated at once that in the absence of methods of cultivating this rickettsia outside its normal host and in the absence of uninfected individuals available for comparison or controlled experiment, there are no criteria for judging whether or not more than one species of organism is involved. In the absence of such criteria and in view of the fact that there are to be found all possible morphological intermediates between the various forms, the rickettsiae of the gonads of *C. pipiens* are treated as a single species. Similarly, there is no basis for determining what are dead, dying, degenerate or "involution" forms.

Forms which may be designated as classical or typical rickettsiae, i.e. tiny coccoids grading into short, straight rods of the same diameter and staining characteristics and occurring singly or in pairs, are always present (Figs. 1–8). These measure in diameter 0·25–0·5μ, the rods 0·5–1·3μ in length. Many elongate and dividing rods stain relatively densely at each pole in contrast to the pale intermediate portion, which may or may not be constricted (Text-fig. A, 1–3). These bipolar densities may be cap-like (Text-fig. A, 18) or coccoid in shape (Text-fig. A, 1, 2). The intermediate area may equal a third or more of the total length. The ends of such rods are typically rounded. One or both ends of the short rods may be drawn out to an ill-defined point. Some coccoids appear as though the material of one side were drawn out to a blunt point, perhaps representing the point of separation at division. This rickettsia exhibits the poorly defined outline characteristic of rickettsiae in general. The shape of both rods and coccoids is irregular. Indeed, it may be difficult in a given field to find any two organisms, except members of a pair, which match each other closely in size, shape and staining intensity. The coccoids are rarely perfectly spherical. The rods may have parallel sides or may be oval in outline. In some rods which appear swollen, there is a surface concentration of the stainable material resulting in a pale interior with sharply stained periphery and cap-like thickenings at either pole. Division of the rods gives rise to rods or coccoids. To what extent continuous division by binary fission takes place cannot be stated, but it may be noted that fields composed exclusively of small rods and coccoids are rare. Characteristic fields and individuals are illustrated in the photomicrographs (Figs. 1–8) and in the drawing (Text-fig. A).

Reference is made at this point to the rather detailed account accompanying the figures, since it has been deemed unnecessary and undesirable with this extremely variable organism to mention specifically in the text all the variants illustrated.

Enlarged coccoids and their relation to other forms.

In nearly every field occur markedly enlarged coccoids measuring 0·6–0·9μ (Figs. 1–8, Text-fig. A, 9, 14, 19–21, 32–35, 40–43) with at times still larger forms 1·0–1·8μ in diameter (Figs. 5–7, Text-fig. A, 22–31), which are referred to respectively as intermediate and large coccoids, in contrast to the small coccoids of the typical rickettsia form. The enlarged coccoids occur singly or in pairs. In both sections and smears they may be surrounded by a halo (Figs. 15, 38, 39). They stain densely, are relatively sharply outlined, and are usually somewhat irregular in shape. A complete morphological series between these and the small coccoids occurs (Fig. 3). Many of the enlarged coccoids exhibit unevennesses of staining which may take the form of an irregular light area at one side or in the centre (Text-fig. A). They never possess a central dense portion grading to a more faintly stained periphery which is often characteristic of the smaller forms. Intermingled with the intermediate coccoids are small, sharply curved or **V**-shaped forms and occasionally a closed ring. Present usually in greater number are more or less compact groups composed of various combinations of rods and coccoids. These forms or groups intermingled with the intermediate coccoids are of about the same total mass as the latter and often approach the same staining intensity. The individuals in such groups are roughly equivalent in mass to from two to four small coccoids, the following representing common examples: a single rod, sharply bent or curved (Text-fig. A, 11–12, 36, 45, 48); two rods, closely apposed (Text-fig. A, 18), **V**-shaped (Text-fig. A, 13, 44) or forming an oval (Text-fig. A, 16) or ring; a rod and one or two coccoids (Text-fig. A, 10, 34) or a tetrad consisting of two pairs of organisms (Text-fig. A, 17). It is believed that the sharply curved individuals and the compact groups are derived directly from the intermediate coccoids. In like manner there are intermingled with and derived from the large coccoid forms spheroidal groups composed of all combinations of rods and coccoids, roughly the equivalent in mass of eight to twenty small coccoids (Text-fig. A, 24, 28, 29). Due to the greater diameter of the original forms the rods may be straight, or if curved, are less sharply so than in the intermediate groups. It appears as though the originally sharp outline of the enlarged coccoids becomes less distinct as the densely stained substance is transformed into a more and more faintly staining matrix containing "daughter" forms which are meanwhile becoming more definite in shape and, as the "parent" matrix disappears, finally become entirely free. On the basis of this interpretation the curved forms so characteristic of this rickettsia would be those which have become free from the restraint of the parent matrix and are in process of straightening out. Division meanwhile



Text-fig. A. Various forms of the rickettsia of *Culex pipiens* from Giemsa-stained smears and sections. Drawn at 10,000 diameters. From ovarian smears, except Nos. 38 and 39 which are from a section of a larva with rickettsiae lying extracellularly in the body cavity. Nos. 1–29, for the most part, drawn from field shown in photomicrograph, Fig. 5.

1–8. "Typical rickettsiae", i.e. small rods and coccoids occurring singly and in pairs.

9, 19–21, 32, 41–43. Enlarged coccoids, intermediate in size, some densely stained throughout (19–21), others with lighter central portions (9, 32) or appearing as rings (41–43).

10, 14, 15, 33–35, 40. Intermediate coccoids, the original outline of which is more or less intact, showing "daughter" forms, of which sharply curved or V-shaped forms and paired rods are common examples.

11–13, 36, 44–49. Curved or V-shaped forms interpreted as having been derived from enlarged coccoids. With the disappearance of the "parental" matrix they tend to straighten out, undergoing elongation and division.

16–18. Paired rods, a very common "daughter" group. They may form ovals (16) or imperfect rings, or may be closely apposed. They may elongate without complete separation (16, 18) or divide to form a tetrad (17).

22–31, 38, 39. Large coccoids with daughter groups in all stages from those exhibiting indistinct punctiform, linear or irregular condensations (22, 23, 25, 30, 31) to those in which rods and coccoids are more or less distinct though still cohering in a compact mass.

9, 14, 15, 21. Enlarged coccoids occurring in pairs, interpreted not as division forms of large coccoids but as the result of enlargement of pairs of small coccoids prior to formation of daughter groups.

37. Filament with several constrictions.

may have taken place, since the more open curved forms usually consist of at least two members. The irregularity of the curved forms is extreme. The diameter and staining intensity may vary among members of the same group. The irregularities of outline are chiefly those of the inner contour; the outer approaches a smooth curve. The view that curved forms are derived from enlarged coccoids is further supported by the fact that curved forms rarely are found unaccompanied by enlarged coccoids, while in those cases where small rods and coccoids predominate, as in many larval gonads, both enlarged and curved forms may be practically absent.

In the case of both intermediate and large coccoids the process of giving rise to "daughter" groups appears to be similar. The nature of this process is not understood and no exact parallel among either bacteria or Protozoa suggests itself. At this time, no specific terms are applied to, nor coined for, the various "stages", and the terms "parent", "matrix" and "daughter groups" are used for convenience. No clear-cut division of the whole body of the enlarged coccoids by either binary or multiple fission can be demonstrated. The process appears to be rather a rearrangement of the substance of the organism into one or more individuals embedded in a matrix which gradually disappears. This interpretation, however unorthodox it may be, appears to accord with the observed facts better than any other. The very numerous morphological intermediates between the enlarged, dense coccoids, those less densely stained in which vague punctiform and linear condensations of stainable material may be made out, and the closely coherent groups of organisms, seem to permit no other interpretation than that these forms are directly related. That the sequence of events may be in the reverse direction, involving the curling up of free rods, or the aggregation of separate organisms, or the cohesion of the products of successive divisions, such forms later fusing into a dense granule with complete loss of identity of the original individuals, seems unlikely.

It would appear that the enlarged coccoids are derived chiefly from the small coccoids. There are to be found, particularly where intermediate coccoids are abundant, numbers of small coccoids which are more sharply outlined and stain more densely than normal, with others distinctly enlarged (Fig. 3). One or both members of a pair may become enlarged. It appears as though the enlarging coccoids fail to divide, with the result that there may be a large proportion of single coccoids of all sizes. It is characteristic also of such fields that the individuals tend to be separated from one another by a clear space or to be surrounded by haloes. This separation may be seen in thicker portions of smears where infected cells have not been greatly distorted, and is in contrast to the closer packing of the rods and curved forms. Later, as the enlarged coccoids give rise to daughter groups, the infected cell still shows the whole mass of organisms distinctly partitioned into tiny, compact groups intermingled with densely stained, enlarged coccoids (Figs. 6, 7). Such fields recall

the masses of "pleomorphic granules" of *Rickettsia ruminantium* described and figured by Jackson (1931).

While the sequence from small coccoids to enlarged forms which give rise to daughter groups embedded in a matrix with subsequent release of small, straight or curved rods and coccoids, is apparently common, it is hardly to be considered comparable to a life cycle. Apparently failure to divide with subsequent enlargement and matrix formation may occur at any time, but with no inevitable series of stages to be completed nor any definite size or point of "maturation" to be attained. The production of daughter forms may occur almost at once, or it may be delayed until the parent coccoid has attained considerable size. The rate of production of daughter individuals may be unequal within the same matrix, some forms being almost free while others are still poorly defined within a densely staining portion of the matrix (Text-fig. A, 28, 29). Growth or division or both may take place before the daughter forms are free. The large coccoids finally produce daughter groups of small rods and coccoids which are distinctive chiefly in that they form a compact group (Text-fig. A, 24–29). The forms derived from intermediate coccoids, however, more sharply reveal the impress of the parental matrix and since these forms are usually numerous, impart to smears of this organism their characteristic appearance. The intermediate coccoids give rise to several forms or groups, of which the following are common examples: (*a*) One may find coccoids only slightly enlarged, which show a light interior with peripheral condensation (Text-fig. A, 9, 15), these being apparently the forerunners of the tiny, sharply curved forms (Text-fig. A, 10–12). The latter are often much more densely stained than the small straight rods. Their outer contour is sharp, while the inner one may merge into faintly staining material filling the open side (Text-fig. A, 15). In such cases the enlarged coccoid has given rise to but a single organism. More commonly, however, one finds at least two organisms produced, either originally or by division before the daughter forms are completely free. Such forms appear as (*b*) compact groups composed of a curved rod and one or two small coccoids (Text-fig. A, 33, 34) or (*c*) as a group of small coccoids (Text-fig. A, 40). (*d*) Pairs of very short rods or minute ovoid forms, their axes at an angle, are common (Text-fig. A, 44). (*e*) Also numerous are the paired rods touching each other at one or both ends or for their entire length (Text-fig. A, 13, 14, 16, 18, 35). These are obviously not chance appositions, since they generally match each other much more closely than they do their neighbours, and in addition faintly staining material may often be seen between them. The paired rods may elongate without complete separation (Text-fig. A, 18) and in this position divide to form tetrads (Text-fig. A, 17).

The various forms described above may be found at all stages of development of the mosquito. In general, the larval gonads contain chiefly straight rods and coccoids, while at about the time of sexual differentiation which occurs in the fourth larval or pupal stage, the proportion of enlarged coccoids,

daughter groups and curved forms increases, and in adults these forms may become very numerous, particularly in hibernating females. There are no differences between testes and ovaries as to morphology or proportions of the forms found in them.

Sections. All the various forms described above may be seen most clearly and in greatest number in smears, but they may also be demonstrated in sections. They exhibit the same variations in intensity of staining in sections as in smears. The smaller coccoids and rods, which are more or less closely packed together, tend to stain faintly but include some which stain densely. Those forms surrounded by a halo usually stain intensely and retain the stain during differentiation (Fig. 15). The haloed forms include single and paired coccoids, intermediate coccoids and various daughter groups. The haloes, usually not more than two or three in any one cell, are spherical and form a clear area 0·7–1·0μ in thickness about the included form or forms. The organisms isolated by their haloes from surrounding forms may be observed with especial clearness. Their appearance is precisely the same as that of corresponding forms seen in smears. While in general only germ cells are infected, exceptions occur. These instances provide unusually favourable opportunities for studying the rickettsiae in sections, since the organisms may be scattered and thus permit clear observation *in situ*. In sections of larvae an occasional cell of a Malpighian tube is found infected. The rickettsiae tend to be separated from one another and are evenly distributed throughout the cell. Small, straight rods and coccoids predominate, but all the other forms are also represented, comparable as to size, staining, etc., with those of gonad smears. In one case a considerable number of organisms were found lying between the fibres of a muscle and in the body cavity immediately surrounding it. This infection was of particular interest since the organisms were extracellular. They differed in no respect, however, from those found in germ cells. The daughter groups were abundant and furnished a particularly clear demonstration of the characteristics observed in smears (Text-fig. A, 38, 39). These unusual infections show that in slightly different environments, and even extracellularly, the organism may exhibit the same characteristics as in its normal habitat. Furthermore, the complete agreement of observations made from both smears and sections adds to the confidence with which smears may be used in studying the morphology of these microorganisms.

Fresh preparations. The various forms exhibited by this rickettsia may, for the most part, be demonstrated also in fresh preparations. In a freshly dissected, uninjured gonad, the germ cells usually exhibit a glassy refractivity with no internal structures to be made out (Figs. 11, 12, 14). On injuring the gonad wall, however, a sudden change in the refractivity may take place, and nuclei and rickettsiae may become at once obvious. The same change in refractivity occurs gradually on allowing the preparation to stand (Fig. 10). The rickettsiae in larval germ cells, which may fill the cytoplasm completely

or form merely a mass at one side, are somewhat refractive and have the appearance of colonies of small bacteria (Fig. 10). After the preparation has stood a short time, some of the rickettsiae, particularly at the edge of a mass, may exhibit Brownian movement, but no true motility has been observed. In adults, and particularly in hibernating females, the resemblance to a bacterial colony is less marked because of the great variety of forms, though the refractivity of the individuals is about the same as that of small rods and coccoids. The enlarged coccoids are ovoid or spheroid when released from the cell. Little of their internal structure, except that they are not homogeneous, can be made out with ordinary illumination. In dark-field preparations, however, nearly all the various forms found in smears are strikingly revealed, the stainable portions being represented by brightly luminous material. The rods and small coccoids are luminous rods and points, with no sharp outline. The enlarged coccoids, particularly the intermediates, are correspondingly large and brilliant. There are also to be found, often in large number, enlarged coccoids which appear as hollow spheroids, part of the surface layer of which is relatively thick and brightly luminous, while the remainder is demonstrable only in optical section as a faintly luminous outline. Such forms, as they move about in the preparation, usually appear in any position as brightly luminous rings, often irregular or imperfect, the breaks being bridged by the faintly luminous material. Such forms are luminous counterparts of the forms which in smears exhibit a more densely stained periphery or appear as imperfect rings and sharply curved forms (Text-fig. A). Dark-field preparations also contain spheroid groups of daughter forms in which the faintly luminous material cannot be made out. Some of the luminous rings, particularly those which are larger and more irregular, are seen to be disks rather than portions of spheroids. Though the whole field of organisms may be in active molecular movement, the rods and occasional short filaments exhibit no flexibility. The members of daughter groups maintain their relative positions and move as a unit. In a few instances large numbers of spherical granules possessed only a faintly luminous surface with dark interior, approaching typical bacteria in this respect. The luminous surface layer was not of uniform thickness, however. These forms may be represented in smears by faintly staining rings which occur occasionally (Text-fig. A, 41, 42). It is not always possible on a given occasion to secure any particular form in adequate numbers, and the dense, homogeneous large coccoids of smears have not been identified with certainty in dark-field preparations. The large coccoids were found in numbers, but the daughter forms were already clearly visible as luminous points or short rods at the periphery of a sphere, though not projecting from the surface. The interior was not luminous. The daughter forms seem to arise in all cases at the periphery. This is most clearly seen, of course, in the large forms, since in the smaller ones daughter individuals though at the periphery would at the same time occupy most of the interior. Peripheral origin is also supported by the number of daughter individuals per group which varies roughly as the

square of the diameter rather than as the cube, which would be the case were the coccoid to be filled solidly with daughter forms.

Haloes or clear areas surrounding individuals or small groups may be seen at times in unbroken germ cells. They appear as spherical vacuoles within which the individual organisms, varying in number from three or four to fifteen or twenty, are in active Brownian movement. In one or two instances the addition of distilled water caused the entire mass of rickettsiae within unbroken cells to be partitioned off into such spherical vacuoles, each with its contents in Brownian movement. The fact that distilled water at other times failed to produce this phenomenon makes it appear that it depends upon the presence of organisms with haloes already formed. Whether the haloes are part of the organism or represent a cytoplasmic reaction on the part of the host cannot be stated.

Histopathology of rickettsia-infected gonads

In Giemsa-stained sections the germ cells at all stages in both sexes may be demonstrated to be invariably infected with the rickettsia. This is the only tissue normally invaded, although occasional exceptions have been found as noted above. The embryo has not been studied, but in newly hatched larvae the cytoplasm of the germ cells is already filled with rickettsiae.

Larval gonad. The paired larval gonads, which lie in the sixth abdominal segment, consist of thin-walled, oval or fusiform sacs filled with germ cells (Figs. 9, 10). The large nuclei of the latter permit ready identification of the organ in sections. In the first larval instar the germ cells may form such a compact mass that cell boundaries are difficult to make out, though in later stages there may be considerable space between germ cells. The gonad wall appears as a thin membrane with small, flattened nuclei on the inner surface. In very young larvae, the nuclei may be the only obvious feature of the gonad wall, but at all stages the gonad exhibits a smoothly rounded contour in spite of the large, irregular germ cells within. A colourless, somewhat refractive membrane may at times be demonstrated in sections. The presence of such a membrane is further evidenced by the fact that during dissection the gonad may become engaged in the surface film of the dissection drop and resist wetting. No rickettsiae have been demonstrated in the gonad wall at any stage.

The thin shell of cytoplasm covering the large nucleus of the germ cell always contains rickettsiae. Where the cytoplasm is thinned by pressure of neighbouring cells, it may contain but a single layer of scattered organisms, while elsewhere the cell may appear to be distended with a mass of them. In a small proportion of the germ cells the rickettsiae encroach upon the region normally occupied by the large nucleus and completely fill the cell except for a small mass of chromatin. These are "pathological" cells which are particularly numerous in the testis and are described below in connexion with that organ. They may occur even in the first instar, and once formed

apparently persist, but undergo no further development as germ cells. Their rickettsiae exhibit the same variety of forms as those of normal germ cells. No intranuclear rickettsiae have been demonstrated at any stage. It is thus seen that all the germ cells are infected from the earliest stages, and that all except the pathological cells transmit their infection at division to succeeding generations of germ cells.

Testis. Late in the fourth larval instar the sex of the gonad may usually be determined. The testis is ellipsoidal in shape and consists of a series of transverse chambers filled with germ cells in various stages of spermatogenesis, proceeding in regular succession from the mass of spermatogonia at the pointed anterior end through the first and second spermatocytes and spermatids to the chambers at the posterior end filled with spermatozoa (Figs. 15–21). The partitions are of tissue similar to and continuous with the thin testis wall, and are free of rickettsiae. The spermatogonia and spermatocytes are similar in size and appearance to the larval germ cells. They are infected with rickettsiae, though some may contain only a few, as though there were failure of the organisms to keep pace with the increase of host cells. No rickettsiae have been demonstrated in the spermatozoa themselves. In smears there are usually associated with masses of immature spermatozoa fragments of cytoplasm containing rickettsiae which doubtless represent the residual protoplasm normally sloughed at this stage. At least this serves to account for the elimination of the rickettsiae from the sperm cells. Spermatogenesis is thus carried on by germ cells invariably infected up to a certain stage, but this condition must nevertheless be considered "normal", since in this species of mosquito there are no germ cells without rickettsiae.

Pathological germ cells.

The pathological cells form a striking feature of nearly every testis. Scattered among the spermatogonia and spermatocytes are a few greatly enlarged cells completely filled with rickettsiae except for small masses of chromatin (Figs. 15–18). There occur still other cells, normal in size, in which the nucleus has become a dense mass of chromatin while the rest of the cell is filled with rickettsiae (Fig. 18). These pathological cells were probably first observed by Heimann (cited by Lomen, 1914) and have been described in some detail by Lomen (1914) and Whiting (1917). The associated rickettsiae remained undiscovered by them and other cytologists, due probably to the indifferent staining of these organisms with haematoxylin and other stains used in chromosome studies. Cholodkovsky (1905), in a paper on the structure of the testis of various Diptera, noted and figured "large nuclei and peculiar round bodies" among the spermatozoa of *Culex annulatus*. It is, of course, unknown whether or not these are related to the rickettsia-laden pathological cells of *C. pipiens*, but the possibility suggests itself.

Several types of degeneration of the male germ cells of *C. pipiens* were described by Lomen (1914) and Whiting (1917) as follows: (1) Lomen de-

scribed germ cells which appeared to fuse with each other and with the testis wall or septa, and which, he believed, probably entered into the formation of such walls. Whiting does not mention such cells nor has the writer observed them. (2) Cells with pycnotic nuclei represented by one or more chromatin masses. These were abundant in Lomen's material but uncommon in Whiting's. They were well represented in the writer's specimens (Fig. 18). (3) Enlarged cells with small chromatin masses and darkly staining cytoplasm. These striking cells Whiting found to be more numerous than the preceding type. He stated that "in these cells the nuclei become very large and take a blue-black stain with iron-haematoxylin. They seem to absorb all the cytoplasm and to swell up to many times the size of the original cells. Very dark masses may be seen in these nuclei". These forms the writer has found to be almost always present (Figs. 15–18). While they are outnumbered by those with pycnotic nuclei, their great size, dark staining, and the fact that one cell runs through several sections and may easily be picked out at low magnification, makes them a conspicuous feature of the testis. The dark staining is due to the rickettsiae and not, as Whiting supposed, to the invasion of the cytoplasm by the nucleus. The rickettsiae in these cells tend to be more densely packed and to exhibit more forms which stain densely than in other cells. The enlarged coccoids, in particular, may stain intensely with Giemsa and relatively well with haematoxylin, and retain their stain well during differentiation. With both stains, cells have been seen in which masses of enlarged rickettsiae stood out more prominently than normal nuclei, but no cells were noted in which these cells would be confused with nuclei. The dark masses observed by Whiting were probably particles of chromatin or groups of enlarged rickettsiae or both. (4) Whiting described as the commonest type of pathological cell in his material one characterized by chromosome division without corresponding cell division. Among the second spermatocytes and spermatids were cells which he interpreted as first spermatocytes in which the tetrads had divided, yielding cells with twelve chromosomes instead of the six or three normal to the diploid and haploid forms respectively. No abnormality of the cytoplasm was mentioned by him. Since in the present study only casual attention has been given to the chromosomes, the writer has no information to offer as to this type of pathological cell. In a few cases there have been found in sperm chambers greatly enlarged cells, still spherical in outline, possessing a number of chromatin masses, uniform in size and about twelve in number, accompanied by other cells of the same appearance except that the chromatin masses were about twice as large and half as numerous. Accurate counts were not made, but the picture suggested the possibility that such cells might have been derived from those described by Whiting, in which abnormal chromosomes, twelve or six in number, persisted as separate masses of chromatin of uniform size.

In addition there are encountered bodies which superficially resemble some of the pathological cells and might at times be confused with them. They arise

in the gonad wall or septa and are the cell inclusions (*NR bodies*) described in section II.

The enlarged pathological cells and those with pycnotic nuclei, the writer believes, are to be regarded as manifestations of the same process. Both are characterized by degeneration of the nucleus and the multiplication of rickettsiae in the remainder of the cell, which may or may not become enlarged. There are abundant morphological intermediates between these two forms. The process is highly variable, and no two individuals nor any two lots of material show the same proportion or distribution of forms. Apparently degeneration may set in at any stage of the larval gonad or adult testis, and such cells or their remains may be demonstrated at subsequent stages. It is possible that the form assumed may depend upon the age of the cell. Obviously the process has a greater period in which to manifest itself in those cells which degenerate in young larvae than in those which have developed normally to the spermatocyte stage. It seems reasonable to assume that the degeneration of these cells is the result of the rickettsia infection. Such cells do not occur in *Culex territans* which lacks the rickettsiae, nor are they known to occur in other mosquitoes.

The pathological cells of the undifferentiated larval gonad are usually of the type characterized by pycnotic nuclei and little increase in size. In the testis, however, both types occur and may accompany germ cells of any stage of development. There may be one to several such cells in some chambers, while others may lack them entirely. However, no testis entirely free of pathological cells has been observed. Those accompanying the germ cells through the spermatocyte stages are usually spherical, sharply outlined and often densely stained (Figs. 15–17). Those intermingled with spermatids and spermatozoa seem to be in the process of breaking down. These "old" cells often appear as irregular masses of faintly staining material containing a loose mixture of rickettsiae and chromatin particles of all sizes (Fig. 20). Lomen believed the cells with pycnotic nuclei to become absorbed, but noted that the enlarged ones persist among the spermatozoa. Whiting does not mention pathological cells beyond the spermatid stage. In the writer's material, both types of cell may be demonstrated with the spermatozoa, but most seem to have become the "old" pathological cells described above. They are usually conspicuous in fresh preparations as one or more rounded bodies embedded in the mass of spermatozoa (Fig. 21). Chambers have been found filled with remains of pathological cells to the complete exclusion of spermatozoa (Fig. 20).

In fresh preparations the pathological cells may appear immediately as masses of refractive rickettsiae, while the normal germ cells are refractive and reveal their rickettsiae only after standing some time (Figs. 12, 14).

Ovary. Soon after differentiation of the larval gonad into the ovary this organ is seen to consist of a large central duct with developing follicles radiating from it, the latter embedded in tissue consisting chiefly of thin layers of cells. The outer wall of the ovary consists of a thin investing mem-

brane, while a second, the inner membrane, surrounds each follicle. Between these two membranes are loosely placed cells, chiefly of tracheal and muscular tissue. The young follicles consist of several large cells, the oogonia, similar in size and appearance to the larval germ cells, surrounded by a closely adherent layer of small cells, the follicular epithelium (Figs. 11, 12, 24). The follicular epithelium is prolonged into a column of cells which joins the epithelium of the central duct, forming a peduncle. As the follicle develops, the oogonia increase in number and a group consisting of seven nurse cells and a single oocyte and surrounded by follicular epithelium, is divided off at the proximal end (i.e. nearest the central duct) to form the primary follicle, leaving at the distal end a small mass of cells, the germarium, which consists of oogonia and small follicular cells which may not as yet constitute a distinct layer. Nurse cells and oocyte increase in size and the follicular epithelium becomes a definite layer of uniform thickness which in sections is often separated from the cells within. This is the "resting" stage of development of the ovary and is that found in unfed and in hibernating females.

The oogonia, nurse cells and oocytes are invariably infected with rickettsiae, which now tend to pack the cytoplasm more densely than at previous stages. On the other hand, no rickettsiae have been demonstrated either in the follicular epithelium or in other tissues of the ovary, i.e. investing and inner membranes, cells of muscles and tracheae or epithelium of central duct. Pathological cells occur, but are uncommon compared to the number in the testis, and have been demonstrated only in the germarium, where they are similar in appearance to the pathological cells of the larval gonad. In fresh preparations it appears at times as though a pathological cell had ruptured, liberating its rickettsiae, which are found in the spaces between the other germ cells (Fig. 12). Rickettsiae in ovarian germ cells exhibit the same range of morphology as in those of the larval gonad and testis. The proportion of large coccoids and correspondingly large daughter groups may be greatly increased in hibernating females, particularly in those which have been kept at laboratory temperature for several weeks (Figs. 5–7). The forms surrounded by haloes are also particularly abundant in the enlarged oocyte of the primary follicle (Figs. 38, 39). At all stages and particularly in the ovary, there are usually a few forms which stain very densely in sections. These are usually slender rods or paired coccoids though all other forms are also represented among these densely stained organisms. They may or may not be surrounded by a halo. In one case there were found large rods staining densely with haematoxylin (Fig. 41). Whether these are exceptional forms of the rickettsia or represent another organism cannot be stated.

The ovary has not been studied beyond the "resting stage", except to demonstrate by means of smears that rickettsiae may always be found in eggs and embryos.

Relationship of rickettsia to host

The role of this rickettsia is that of a relatively harmless parasite, which, however, approaches the status achieved by the many intracellular symbionts[1] of insects, in that it infects all individuals of a given species and is invariably transmitted via the egg, but falls short of that status in that it apparently causes to a variable degree the degeneration of certain host cells.

Some phases of the problem of classifying the rickettsiae

It is not the purpose to present here a detailed consideration of the whole question of classifying the rickettsiae, but rather to call attention to some phases of the problem suggested by the present morphological study.

The writer believes that the characteristics held in common justify the view that, in spite of the diversity of organisms included, the rickettsiae constitute a group more closely related to one another than to any other group. That they are allied to the bacteria is possible from the fact that they usually appear as rod and coccoid forms multiplying by binary fission. On the other hand, Neitz, Alexander & du Toit (1934), in tentatively proposing the union of the genera *Anaplasma*, *Grahamella*, *Bartonella* and *Eperythrozoön* to form the *Anaplasmidae* as a family of the *Haemosporidia*, noted that these organisms have features in common with the rickettsiae, but considered the latter more distant in relationship because of their affinity for endothelial cells, while the former are blood parasites. This view takes account only of the few pathogenic rickettsiae. It may further be pointed out that the four genera of blood parasites include species which are transmitted by arthropods. When the arthropod phases have been studied, a comparison with the rickettsiae will be on a sounder basis. The relationship of rickettsiae to bacteria or other groups must be considered at present uncertain.

In cultivation studies on the well-known bacteroids or bacterial symbionts of cockroaches, Glaser (1930, 1930 *a*) included these organisms with the rickettsiae. The writer shares the view of Kligler & Aschner (1931) that they are clearly not rickettsiae. They are relatively large, Gram-positive, sharply outlined, bacterium-like organisms which stain intensely with ordinary dyes. Glaser obtained in cultures, made from the fat body of two species of cockroaches and in one case from a faecal emulsion, organisms which he has placed in the genus *Corynebacterium*, and which he considered to be the bacteroids of these cockroaches. Thus, in his view, his results represented the successful cultivation of certain rickettsiae. Although they may serve to classify the

[1] The term *symbiont*, as it is customarily employed in the extensive literature of the intracellular symbionts of insects, refers rather to the invariable association of insect and microorganism and to the exquisite equilibrium which characterizes it, rather than to proven mutualism. The use of the term *symbiont* in the absence of demonstrated mutualism has been criticized under the misconception that de Bary (*Die Erscheinung der Symbiose*, Strassburg, 1879) defined *symbiosis* as strict mutualism. Actually, he used it as a general term which specifically included parasitism and commensalism as well as mutualism. The confusion in modern usage makes desirable the definition of the terms *symbiosis* and *symbiont* whenever they are used.

blattid bacteroids, the writer considers that they have no immediate bearing on the problem of the rickettsiae.

The only rickettsiae which have been cultivated on artificial media are the extracellular rickettsiae of the Pupipara (Kligler & Aschner, 1931). These organisms form a closely related group, the precise relationship of which to other rickettsiae may be left an open question.

In the absence of methods of studying the great majority of rickettsiae outside their normal hosts, a consideration of certain morphological phases may be profitable. The lack of sharp outline and faintness of staining with ordinary dyes which characterize the entire group may indicate a body structure distinct from that usually encountered in bacteria. Furthermore, with dark-field illumination some rickettsiae appear as luminous points or rods, while bacteria generally present a luminous outline with dark interior. It is possible that the dark-field appearance may prove to be a distinctive characteristic of the rickettsiae. The extracellular rickettsiae of the Pupipara exhibit indefiniteness of outline and faint staining but in culture approach more closely to bacterial characteristics. References to the dark-field appearance of these organisms, either in their insect hosts or in culture, have not been found in the literature. It may be noted that the "elementary bodies" of psittacosis, which closely resemble rickettsiae, also appear as luminous points with dark-field illumination (Bedson & Bland, 1934).

Forms of rickettsiae other than typical rods and coccoids.

A number of rickettsiae are characterized by forms or stages which depart widely from typical rods or coccoids in morphology but which can hardly be set aside as mere degeneration forms, since in some cases they are very numerous, occur in uniform masses or are apparently multiplicative forms. They present problems in morphology the solution of which is essential to an understanding of the nature of the rickettsiae. In this connexion there may be cited the ring forms of *Rickettsia ruminantium* (Jackson, 1931), the ring and other forms of *R. conjunctivae* and of another rickettsia-like organism found in the conjunctival epithelium of sheep and goats respectively by Coles (1931, 1935), the ring and C-shaped forms found in the bedbug (Hertig & Wolbach, 1924), and the ring and other forms derived from enlarged coccoids of the mosquito rickettsia herein described. The South African investigators have not described the stages by which rings may be derived from rods and coccoids nor the reverse process, though Jackson (1931) mentioned that the larger rickettsia granules assume a variety of irregular shapes and that there are found all intermediate forms between rings and aggregations of coccoid forms. Hertig & Wolbach (1924) considered it probable that the small rings and C-shaped forms, and sharply curved rods of the bedbug rickettsia are derived from, and indeed are formed within, the discoid or ovoid granules and that their shape reflects the restraint imposed by the body of the parent granule. Similar restraint was postulated in the case of the occasional "filament balls",

which were tentatively identified with large, refractive, spheroid bodies found in fresh preparations. No membrane as a separate structure was noted, but it appeared rather that a structureless matrix enclosed each filament.

The various forms just discussed appear to have in common the characteristic that within a relatively large spheroid, ovoid or discoid structure, there occurs a concentration, usually peripheral, of stainable substance. Both the densely and faintly staining portions usually take the same colour with Giemsa's stain, i.e. shades of red and purple. On the basis of the present study of the mosquito rickettsia, it seems probable that such condensations finally assume the size, form and staining characteristics of the smaller rods or coccoids characteristic of the particular organism, and that the faintly staining portions of the original body disappear and thus release the included form or forms, which may or may not grow or divide during the process. Such a sequence of events might in some cases account for the compact aggregations of rods and coccoids, **C**-shaped, sharply and irregularly curved rods, etc., encountered throughout this group of organisms.

The writer wishes to call attention to the fact that ring forms similar in appearance to those discussed above are also known in organisms not classified as rickettsiae, namely, some of the intracellular "bacterial" symbionts of the Pupipara, notably those of *Melophagus ovinus*, *Hippobosca camelina* and *H. capensis* (Aschner, 1931); the intracellular "bacterial" symbionts of *Cimex hirundinis* (Pfeiffer, 1931); and the "unknown organism" in the conjunctival epithelium of the goat in South Africa (Coles, 1935). The organisms belonging to the genus *Eperythrozoön* characteristically are delicate rings 0·5–1·0μ in diameter, which represent apparently the more densely stained periphery of a disk. These blood parasites also exhibit a variety of other forms including ovoid, rod, dumbbell and comma-shaped forms. Certain forms in a preparation of the *Eperythrozoön* of the mouse, kindly furnished by Dr David Weinman, as well as the figures of *E. ovis* of the sheep given by Neitz, Alexander & du Toit (1934), bear a marked similarity to corresponding forms of the rickettsia of *Culex pipiens*. In calling attention to the ring forms of symbionts and Eperythrozoa the writer does not wish to imply that these are related to rickettsiae, but has in mind rather that they exhibit a structure uncommon among micro-organisms and that their precise morphology and relationships to other more "orthodox" forms of the respective organisms have not been thoroughly studied.

Rickettsiae which form compact aggregations. While most rickettsiae tend to be distributed throughout the cytoplasm of infected cells, *Rickettsia ruminantium* characteristically forms compact aggregations which do not fill the host cells (Cowdry, 1925), and which even in smears may appear as intact masses (Jackson, 1931). This growth habit may be due to the "chromophobic substance" which forms haloes about individuals and permeates masses of this organism (Jackson, 1931). The intranuclear forms of the spotted fever rickettsia in tissue culture often occur as spheroid, compact masses which

may be difficult to resolve into individuals (Pinkerton & Hass, 1932). A definite halo surrounds such masses. The formation of compact clumps also characterizes *R. rocha limae* (da Rocha Lima, 1930). The rickettsia of *Phlebotomus vexator* (to be reported elsewhere) at times forms sharply circumscribed oval masses which do not fill the host cells and which may be found intact in smears. At times such masses possess a matrix which stains in the fresh condition with neutral red.

The problem of subdividing the rickettsiae.

It may readily be granted that the rickettsiae are too diverse to be included in the single genus *Rickettsia*. Da Rocha Lima (1930) favoured restricting the genus to include only *R. prowazeki*, *R. quintana*, *R. melophagi*, *R. rocha limae*, *R.* (*Dermacentroxenus*) *rickettsi*, and *R. ruminantium*. This grouping well exemplifies the difficulties encountered in classifying the rickettsiae, since among da Rocha Lima's six species are represented almost every characteristic as to morphology, pathogenicity, relationship to host, etc., which has been suggested as a basis for subdividing the rickettsiae. With members of this "narrow" group as precedents, almost every other rickettsia could also be included. At the other extreme, arguments could be advanced for placing in separate genera most of those rickettsiae which have been relatively well studied. The solution of the difficulty undoubtedly lies between these extremes and will require the erection of several genera. The writer believes that for most of the rickettsiae an adequate basis for a general subdivision is at present lacking, but that the division should be undertaken by those working at first hand with the various organisms, as the increase of information may warrant.

In the case of the rickettsia of *Culex pipiens*, the writer believes that the morphological characteristics of this organism justify naming it as a new genus within the group of rickettsiae. There is accordingly proposed the name *Wolbachia pipientis* (gen. et sp.n.), the generic name being in honour of Dr S. Burt Wolbach, the specific name referring to the only known host. This organism is considered one of the rickettsiae, though the limits neither of the whole group nor of the genera *Rickettsia* and *Dermacentroxenus* are here defined nor are other subgroupings as to either families or genera proposed. It seems desirable as well as convenient to continue the use of the term rickettsia in the broad sense previously defined.

The classification of Wolbachia pipientis (*gen. et sp.n.*).

The classification of the mosquito rickettsia may be outlined as follows:

The rickettsiae

A group not precisely definable at present, but which includes members of the genera *Rickettsia* da Rocha Lima, *Dermacentroxenus* Wolbach, and other species, mostly unnamed, occurring usually intracellularly in arthropods. They are Gram-negative, bacterium-like micro-organisms that measure usually less

than 0·5μ in diameter, lack a sharply defined outline, and stain well with Romanowsky stains, but poorly with common bacterial dyes.

Wolbachia (gen.n.)

Generic characters. Micro-organisms having the general characteristics of the rickettsiae, and exhibiting not only minute, bacterium-like forms appearing with dark-field illumination as luminous rods and points, but also enlarged forms within the body of which are contained one to several smaller individuals. Type and only known species, *Wolbachia pipientis*.

Wolbachia pipientis (sp.n.)

Specific characters. *Wolbachia* exhibiting enlarged forms, usually 0·5–1·3μ in diameter, containing individuals which appear as rings, curved or bent rods, or compact aggregations composed of various numbers and proportions of rod and coccoid forms.

Occurrence. In cytoplasm of germ cells of *Culex pipiens*; distribution apparently that of host. *Type locality*, Boston and vicinity.

Synonym. The rickettsia (unnamed) of *Culex pipiens*, Hertig & Wolbach (1924).

The only other rickettsia which on the basis of present information might also be included in this genus is *Rickettsia lectularia* of the bedbug, in which the enlarged granules, rings, **C**-shaped forms and filament balls seem to represent phenomena comparable to those of *Wolbachia pipientis*. However, uncertainties as to the interrelationships of the various forms found in the bedbug have been introduced by the work of Pfeiffer (1931) who believed that he had established that there are two organisms present, one a "symbiont" and the other a "typical rickettsia". While the possibility that there may be two or even more organisms in the bedbug may be granted, the writer does not find convincing Pfeiffer's separation of the various forms, and believes the matter should receive additional study.

II. Cell inclusions (NR bodies) of the gonads of *Culex pipiens* which may simulate both masses of rickettsiae and the inclusion bodies of certain viruses

When freshly dissected gonads of *C. pipiens* at any stage of development are placed in saline to which a little neutral red has been added, there appear immediately variable numbers of brilliantly stained bodies, usually spheroid, which at first glance convey the impression that an occasional germ cell takes the stain (Figs. 9–14, 22–27). These bodies, when the preparation is first made, are often homogeneous, but on standing, the red colour fades and they become uniformly and finely granular. Others may be granular to begin with. In a study of the rickettsiae invariably present in the gonads of the sandfly, *Phlebotomus vexator*, to be reported elsewhere, the writer has found in excep-

tional instances large rounded masses of rods embedded in a matrix stainable with neutral red. These occur particularly in the testis wall, in which the rickettsiae are also abundant. Such rods are not to be distinguished from the normally present rickettsiae. Neutral red preparations of *Culex pipiens* were first made with the purpose of determining whether or not matrices similar to those of *Phlebotomus* were associated with other insect rickettsiae.[1] The constant and striking presence of apparently similar bodies in *Culex pipiens* associated with its rickettsiae, together with failure to demonstrate either matrices or rickettsiae in another mosquito, *C. territans*, at first made it appear that the phenomena in *Phlebotomus vexator* and *Culex pipiens* were related. But, whatever may be the relationship of matrices to rickettsiae in *Phlebotomus*, it is necessary at present, for reasons which are discussed below, to consider them separate entities in *Culex pipiens*. In the mosquito, the red-staining bodies do not occur in the same tissue as the rickettsiae and are distinguishable from rickettsiae by their appearance in fresh preparations and by their staining reactions in sections. They resemble in some respects certain virus inclusions, notably those of psittacosis, but the nature of these bodies in *C. pipiens* is unknown. They will be referred to by the term *NR body*, from their affinity for neutral red.

It may be noted at the outset that there are in insect tissues many granules, spheres and other bodies with an affinity for neutral red. The Malpighian tubes and fat body often contain great numbers of such bodies. The gonads of a number of Diptera, including among those examined mosquitoes of several genera and various simuliid, muscid, mycetophilid and other flies, usually contain a sprinkling of small, refractive granules stainable with neutral red. Most of these granules are in the gonad wall though at times they lie in the spaces between germ cells and occasionally in the cytoplasm of the latter. In some species of mosquitoes it is often found that each germ cell, or each germ cell of a given stage, e.g. first and second spermatocytes, contains in its cytoplasm one small, red, homogeneous, non-refractive body, 1 or 2μ in diameter, which is usually single but may consist of two subspherical portions or of several smaller granules. They have been seen chiefly in *Culex territans* but also occur in *C. pipiens*, *Aedes aegypti*, and *Anopheles punctipennis*. The relationship of the small granules in the gonad wall of various mosquitoes to the NR bodies of *Culex pipiens* is not known, nor, indeed, is it possible in this mosquito to draw a dividing line in the series of forms from the large NR bodies, unmistakable both in the fresh condition and in sections, to the tiny granules for which there is no recognizable equivalent in sections. The term *NR body* is therefore restricted, in the absence of other distinguishing criteria, to those neutral-red-staining bodies of the gonads of *C. pipiens* for which equivalents may be found in stained preparations.

[1] Brief examination of a few specimens of *Cimex lectularius* and *C. pipistrelli* show that gonads and mycetomes contain numbers of bodies which stain with neutral red. They have not as yet been studied further.

The neutral red solution is made by adding a drop or two of a saturated alcoholic solution of the dye to 10 c.c. of 0·85 per cent. saline. Accurate measurement is unnecessary, since solutions varying from pink to distinct red, as seen in a test tube, yield the same results if additional time is allowed for the weaker solutions to act.

Entirely comparable results are obtained with brilliant cresyl blue (one drop of the 0·5 per cent. saline stock solution in 10 c.c. normal saline). The reaction is equally specific, the staining takes place promptly, fading occurs and the NR bodies become granular precisely as with neutral red. The blue colour is less intense than the red, however, and where the two dyes are used simultaneously the red predominates. While the two dyes may be used interchangeably for the study of NR bodies, most of the observations in this study have been made with neutral red.

Janus green, Niagara blue BX, and toluidine blue in dilutions which give a distinct blue colour as seen in a test tube, failed to stain the NR bodies, as did also dilute Lugol's solution (seven drops per 10 c.c. saline). The NR bodies do not blacken when exposed to osmic acid vapour.

Distribution of NR bodies in gonads

In neutral red preparations of larval gonads the NR bodies are particularly striking. The whole gonad with its simple structure lies within a single oil-immersion field, and the large, red bodies, even though only one or two are present, stand out in brilliant contrast to the unstained tissue (Figs. 9, 10, 22, 23). The large NR bodies, which measure approximately from 7 to 12μ in diameter, appear to lie among the germ cells, but close examination shows them always to be peripheral, i.e. they are in contact with or lie very close to the gonad wall. Tiny, irregular red granules may also be found sprinkled singly or in clusters throughout the gonad wall. Some of these smaller forms, about 3μ in diameter, cause the gonad wall to bulge inward. Still larger forms, 5–8μ in diameter, though several times the volume of the original cell, may still be enclosed by the now greatly stretched cell membrane and project further inward among the germ cells (Fig. 19). Most of the larger NR bodies, however, appear to be free of the gonad wall. The foregoing illustrates the typical relationship of the NR bodies to the tissue in which they originate in both larvae and adults, namely, that they arise in, distend and may ultimately become released from, the cytoplasm of cells which form a thin layer, at times little more than a membrane, containing scattered nuclei. The latter are apparently not affected by the NR bodies, mechanically or otherwise. The nuclei do not stain with neutral red and are not concerned at any stage with the production of NR bodies, nor is there any evidence that the germ cells ever give rise to them.

NR bodies of all sizes are to be found in gonads at all stages after sexual differentiation. Their position is similar to that in larval gonads in that they arise in tissue surrounding germ cells and not in these cells themselves.

In the testis the NR bodies are found in or in close contact with the outer wall and septa (Figs. 14, 16, 18, 19). They usually occur in the anterior end with the developing germ cells but are occasionally found in great numbers in the walls of sperm-filled chambers. The NR bodies may occur singly or in patches containing forms of all sizes, some in close contact with each other (Figs. 14, 27).

In the ovary the commonest and most typical NR bodies are those in the follicles rather than in the surrounding tissue. In the larval or pupal ovary NR bodies are found in the thin layer of cells which enclose the oogonia and give rise to the follicular epithelium (Figs. 11, 12). After formation of primary follicle and germarium they are usually limited to the latter. Here their appearance and development are precisely as in the larval gonad. They arise in and distend the thin outer layer of cells and later project among the oogonia (Figs. 11, 12, 24). The entire ovary, though composed of 75–100 or more ovarioles, may contain only two or three NR bodies, with ten to fifteen a common number, while more than thirty or forty per ovary, except those in the pathological follicles described below, is unusual. The majority occur singly, though two and rarely five or six may be found in the same germarium.

NR bodies are found occasionally in the epithelium of the central duct, projecting into its lumen. In hibernating females they may occur in the stalk of cells joining the primary follicle to the central duct. In hibernating females, though rarely in those from summer generations, ovarian tissue other than that of the follicles may contain considerable numbers of large, red bodies and clusters of smaller ones which have not been identified with certainty, in view of the occurrence of similar bodies in such situations in other mosquitoes.

Pathological follicles

In females caught late in the fall or taken from hibernating quarters, there are frequently found primary follicles which contain a large number of typical NR bodies (Figs. 36–40). The latter may occupy only the follicular epithelium, which is greatly thickened, or in extreme instances may replace in whole or in part the nurse cells and oocyte within. It may be impossible to recognize any host cells, either of the epithelium or of the germ cells. Figs. 36 and 37 show such a follicle in a neutral red preparation which, in addition to those NR bodies identified by the stain, consists of spheroid bodies and granular material which may represent rickettsiae, cellular debris or unstained NR bodies. The condition is clearly a degenerative one and such follicles are termed *pathological follicles*. Whether the NR bodies cause or merely accompany this condition is unknown. There may be from one to several such follicles with rarely more than eight or ten in any ovary, though in one instance nearly every follicle was more or less affected. Nicholson (1921) stated that some follicles of *Anopheles maculipennis* normally degenerate but details of the process were not given. It has not been determined whether or not pathological follicles similar to those of *Culex pipiens* occur in other mosquitoes.

They have not been observed in *C. territans*, but no hibernating females of this species have been available.[1]

The process of degeneration of the follicle may be outlined as follows: The NR bodies appear in numbers in the follicular epithelium, which may be greatly thickened; the layer of NR bodies increases in thickness; the epithelium becomes no longer recognizable; and the mass of NR bodies increases at the expense of the germ cells within and may finally replace them.

Morphology of NR bodies

NR bodies in neutral red preparations. In a freshly made neutral red preparation, most of the NR bodies are a deep red, somewhat refractive, and appear to consist of a homogeneous substance which takes the stain uniformly. They are commonly about 6–8μ in diameter but may be as large as 12μ with occasional extremes of about 15μ. They are rarely perfectly spherical and may be ovoid. Irregularities of outline are smoothly rounded. The red colour fades gradually and after a variable period, usually within half an hour, the whole substance appears uniformly and finely granular, becoming more distinctly so as fading progresses, until at the end of 1 or 2 hours there may be no trace of red and the NR body has become a mass of somewhat refractive granules. These are often so closely packed that no details of the granules may be made out, and the outline of the whole mass remains sharp. In some cases swelling takes place, the definite outline is lost and the resulting granular mass very closely resembles the neighbouring masses of rickettsiae. The granules, which include rods, are of about the same size and refractivity as the rickettsiae. The process of fading and becoming granular is usually hastened by the light or heat incidental to photography so that a deeply stained body may fade before a second exposure can be made. In any case, the fading proceeds at an unequal rate throughout the preparation and some may be red an hour or two after others have completely faded. In addition to the homogeneous bodies which stain so deeply, there may be found some which are already slightly granular. The latter stain only pink and fade quickly. Still others are distinctly granular to begin with and do not stain at all. With dark-field illumination the NR body granules appear as luminous points, and are similar in this respect to the rickettsiae, and also to the elementary bodies of the psittacosis inclusions (Bedson & Bland, 1934).

The larger NR bodies may be recognized in saline preparations without the aid of neutral red. Their refractivity is slightly different from that of the germ cells and their colour may also be very slightly different, since at times they possess a faint yellowish cast in contrast to the bluish colour of the refractive germ cells. As in neutral red preparations they are usually optically homogeneous at first but become granular after a short time. The slight

[1] *Culex territans* has never been taken from cellars in which hundreds of *C. pipiens* may be found, though egg-rafts of both species may be collected in about equal numbers from nearby breeding places until late in the season.

difference in colour in addition to the fineness of the granules usually serves to distinguish these bodies from the pathological cells filled with rickettsiae with which they are the most likely to be confused. The pathological cells, it may be noted, do not at any stage stain with neutral red.

Certain general stages may be recognized in the development of NR bodies, as follows: Optically homogeneous bodies, readily stainable with neutral red, gradually lose their affinity for the dye and become granular. They finally appear as sharply outlined, granular masses with no affinity for the dye, or as less compact masses of distinct coccoid or rod-shaped granules no longer possessing a definite matrix.

Morulate NR bodies. In hibernating females many NR bodies display gently rounded prominences and are seen to be divided into vaguely outlined spheres 2 or 3μ in diameter. These forms recall the morulate bodies of psittacosis (Bedson, 1933). The outlines of the internal divisions become clearer as they fade and become granular, or with application of pressure to the coverglass. Whether or not NR bodies break up into a number of smaller forms is not known, but this appears possible. Fresh preparations have furnished no clear indication of this process, though in one instance a germarium contained a mass of about thirty small, red, granular bodies. However, in the sections shown in Figs. 38 and 39 some of the many NR bodies are clearly divided into smaller, densely stained bodies of uniform size, while in others the subdivisions are in turn composed of smaller granules. It thus appears that the final state of the NR bodies, namely, a mass of granules, may be achieved directly or through an intermediate, morulate stage.

Atypical NR bodies. In addition to what may be termed typical NR bodies, to which the foregoing description applies, there are also encountered a number which are atypical as to form, staining reaction or internal consistency. Adult gonads may exhibit double (Fig. 25), trilobed (Fig. 38), or irregular bodies (Figs. 26, 37), which are atypical in form only. Some NR bodies contain vacuoles which stain a lighter shade of red. While usually the matrix appears to be the stainable substance, the reverse condition may occur in which discrete, red, coccoid granules are unevenly distributed in a homogeneous pink matrix.

NR bodies in fixed and stained preparations. The NR bodies are easily recognized in sections by their size, shape and position in the tissues and by distinctive staining reactions with both haematoxylin and Giemsa. The various forms found in fresh preparations are faithfully represented in sections. With haematoxylin they stain with varying degrees of intensity but they may be the most densely stained structures in the entire gonad (Figs. 38, 39). The granular forms appear as masses of dark granules in a more lightly stained matrix. In Giemsa-stained sections the NR bodies and their granules are blue, or occasionally greenish, in contrast to the reds and purples of the rickettsiae with which they might otherwise be confused. In those cases in which the granules are distinct, they appear as a loose mass of well-stained rods and

coccoids distinguishable from neighbouring rickettsiae only by their blue colour (Figs. 16, 19, 40). Sections reveal the probability that at least some of the small red granules found in neutral red preparations are NR bodies. Occasionally there have been found closely associated with groups of larger NR bodies, scattered small forms, the granules of which were identical with those of the larger bodies. The numerous red granules of fresh preparations, however, are not represented by correspondingly numerous bodies in sections and as a result it is not known whether they are related to the NR bodies or not.

The morphology of the NR bodies in the pathological follicles is extremely variable and a single follicle may contain representatives of most of the forms which these bodies exhibit throughout their development. In Fig. 40 the thick layer of NR bodies which has replaced the follicular epithelium contains mostly small forms, some quite irregular in shape. Several exhibit vacuoles in a homogeneous matrix, while others are quite granular. The variety of forms, including trilobed and morulate bodies, shown in Figs. 38 and 39, has been referred to above.

The study of NR bodies in smears is made difficult by the fact that they are relatively so few that they are hard to find, and further that in smears they lack the distinctive staining reaction shown in sections. Teased hanging-drop preparations, in which individual cells and NR bodies may be studied before and after staining, provide a certain, though laborious, means of identifying NR bodies in smears (Fig. 13). Those NR bodies which have not been freed from gonad tissue in making the smear, stain a dense blue with Giemsa and no details of inner structure can be made out. Free, intact NR bodies also stain very densely but are reddish purple. No internal details can be made out except that, at the periphery, coccoid granules may be resolved, a trifle smaller than the surrounding rickettsiae but similar in staining reaction. After prolonged differentiation, or in a smear which has faded somewhat on exposure to light, the entire NR body is seen as a mass of reddish granules, smaller and more closely packed than rickettsiae in germ cells, and embedded in a greyish or pinkish matrix, in contrast to the clear blue of the germ cell cytoplasm. The rapid fading of the neutral red stain in teased preparations together with the considerable granular debris make uncertain the recognition of fragments of NR bodies or isolated granules derived from them. In those cases where fields of small red granules were noted and sketched, equivalent bodies were either not found at all after staining, or else the numerous free rickettsiae prevented identification of individual granules noted before staining. While this method provided several excellent stained preparations of NR bodies, the considerable time and effort expended failed to provide a satisfactory variety of forms.

Smears of pathological follicles were then made in the expectation that at least some of the numerous NR bodies would be recognizable, in spite of the rickettsiae inevitably included. The neutral red stain made it relatively easy to separate a pathological follicle from the rest of the ovary. This was then

dragged out of a small dissecting drop with the needle point so that a minimum of fluid accompanied the tissue. In a successful smear the red follicle disappeared in a tiny pink streak as the needle advanced.

Such smears stained with Giemsa show a number of isolated NR bodies. They tend to resist flattening and are densely stained or even opaque, in contrast to the germ cells of normal follicles which are fragile and permit cytoplasm and rickettsiae to flow out in a thin film. Where flattening or injury to the NR body has occurred, a matrix in addition to the granules is usually obvious. In some cases the matrix appears as though it had cracked in spreading or drying (Figs. 28, 29), indicating a considerable degree of cohesiveness. In such cases the matrix may stain so densely that it obscures the included granules. In other cases the matrix appears as wisps of material containing tiny granules. The latter are mostly coccoid and tend to be of uniform size and closely packed in the matrix, though rods, rings and curved forms also occur. Some stain very faintly and lack definite outline, while others stain densely and are sharply outlined. In smears the granules do not take the blue stain so characteristic of them in Giemsa sections, but are of the same shades of red and purple as the rickettsiae. As a result they reproduce a typical rickettsia picture differing from that of the germ cell rickettsiae chiefly in the preponderance of the very minute coccoids and the presence of the cohesive matrix. It is impossible to distinguish isolated NR body granules from the rickettsiae. The coccoids average smaller than the rickettsiae and measure mostly about 0·2–0·3μ, but include forms as large as 0·5 and 1μ. They may occur in pairs, suggesting division forms. In some NR bodies the granules, particularly the larger ones, are surrounded by a clear area, and such forms with haloes may be common in smears of pathological follicles (Fig. 32). Smears stained with haematoxylin proved unsatisfactory since the granules stained only faintly.

Incidence of NR bodies in different mosquito strains

The incidence or development of NR bodies may vary with the strain of mosquito. Among seven batches of larvae from as many egg-rafts collected at the same time and place and reared under apparently identical conditions, larvae of all ages selected at random contained from one to several typical NR bodies with the exception of those from one egg-raft. In thirteen larvae of this lot only one showed typical NR bodies, two contained small, and hence doubtful, forms, and there were none at all in the remaining ten, though there occurred the usual sprinkling of small red granules in all. Adults from this lot, however, contained NR bodies in normal numbers. The rickettsiae were normal throughout. In larvae from another egg-raft NR bodies were consistently above the average in number. In another lot, normal as to both rickettsiae and NR bodies, there occurred in nearly every larva numerous vacuoles in the germ cell cytoplasm. The majority ranged in size from about 2–5μ but some were as large as a normal germ cell and caused displacement of the nucleus. They were colourless in the fresh condition, did not stain with

neutral red and in sections appeared as sharp vacuoles. In this lot of larvae red granules in the cytoplasm of the germ cells were particularly numerous. No attempt was made to propagate strains to determine whether their various peculiarities would persist through subsequent generations.

Nature of NR bodies

The evidence at present does not permit a decision as to whether the NR bodies and their granules are living agents or are merely some of the great variety of cell inclusions, as yet little studied, which are encountered in most insect tissues. Their appearance and behaviour, however, are consistent with their being living agents or the manifestations of living agents, existing in much the same relation to the host as the rickettsiae. They arise in one tissue, the gonad wall, throughout the life of the host and appear to follow a definite course of growth and development. The matrix possesses an affinity for neutral red and brilliant cresyl blue, as do also the inclusions of psittacosis and certain other virus diseases. They ultimately form a mass of granules simulating in staining reactions and dark-field appearance, rickettsiae on the one hand, and elementary bodies of certain viruses on the other. Their position in the ovary would provide an obvious means of transmission to the progeny via the egg. While demonstrable in nearly every individual, they are greatly variable in number and distribution. Their incidence may vary with different strains of mosquito. Finally, they are associated with a degenerative process of frequent though irregular occurrence, in which they increase enormously in number and replace normal cells.

The possibility that the NR bodies are a stage in the development of the associated rickettsia must be borne in mind, though apparently NR bodies and rickettsiae are entirely independent of one another. The determination of the nature of the NR bodies would, of course, be facilitated by the finding of similar bodies and associated lesions in other insects or by the discovery of strains of *Culex pipiens* which lack them and would therefore be suitable for experimental study.

Inoculation and cultivation experiments

Attempts have been made to cultivate the rickettsiae and NR bodies and to infect mice, but with negative results. Ovaries of hibernating females were used as inoculum in all cases. Legs and wings were cut off and the body placed on a slide so that the posterior end lay touching a drop of saline. While the thorax was held with one needle, traction was applied to the tip of the abdomen with another in such a way that the posterior segments were drawn into the drop of saline partly withdrawing, but not breaking, the intestine. This operation usually caused the ovaries to be withdrawn so that they could be freed completely by cutting the oviducts. They were then washed by transferring to successive drops of saline in a petri dish. No attempt was made to sterilize the surface of the mosquito, though slides, needles and saline were

sterile. This method, which avoids possible damage by sterilizing agents to the micro-organisms in the ovaries, resulted in surprisingly little contamination.

Five chick embryos, in the tenth to fifteenth days of incubation, were inoculated on the chorio-allantoic membrane with whole ovaries or suspensions of ovarian tissue or both. The embryos were held at 37°C. after inoculation. A rapidly growing bacterial contaminant was found in one case in which the embryo had died. The other four remained completely negative. Transfers from the latter to culture media, chiefly NNN medium and serum agar, likewise remained sterile. Opacities were noted in the membrane in the field of operation and some corresponded to the site of inoculation. Examinations of such opacities in fresh preparations, smears and sections were completely negative for micro-organisms. Similar opacities were noted in control embryos.

White mice were inoculated with suspensions of ovary, thirteen intraperitoneally and thirty intracerebrally. In the case of a few which died or became moribund within a few days after inoculation, suspensions of their organs were inoculated into other mice. However, no symptoms or mortality referable to the mosquito organisms were noted, either in the original mice or in those subinoculated. Smears of organs were likewise negative. The survivors of those originally inoculated, seven of the thirteen and twenty-one of the thirty, were apparently normal when killed at the end of two months.

Six tubes of NNN medium or serum agar were inoculated with ovarian tissue and held at room temperature. Three remained sterile while in the others one or two colonies of bacteria appeared. These culture attempts have been as yet too few to indicate anything beyond the fact that in a fair proportion of cases ovarian tissue free of bacterial contaminants may be secured.

SUMMARY

The invariable presence of a rickettsia in the gonads of the mosquito, *Culex pipiens*, is confirmed. This rickettsia is found in the cytoplasm of every germ cell in both sexes at all stages of development from larva to adult. No other species of mosquito is known to be similarly infected.

The morphology of the organism shows the following features. In addition to "typical rickettsiae", i.e. minute rods and coccoids which multiply by binary fission, there is a variety of other forms which are interpreted as a series of stages, usually in the following sequence: small coccoids become enlarged, and instead of dividing by binary or multiple fission, undergo a condensation of their stainable substance into points or rods which ultimately appear as various combinations of sharply curved rods and coccoids retained as in a matrix within the body of the original or "parent" coccoid. As the matrix disappears, they become the irregularly curved forms and compact aggregations of "daughter" forms characteristic of this rickettsia.

The pathological germ cells previously noted in the testis by cytologists were found to occur in both sexes at all stages, and to be apparently the result of infection with this rickettsia.

The problem of classifying the rickettsiae is discussed. The similarity of the rings and curved forms of various rickettsiae to corresponding forms, as yet little studied, in other groups of organisms is pointed out.

The name *Wolbachia pipientis* (gen. et sp.n.) is proposed for the rickettsia of *Culex pipiens*.

Large, homogeneous or granular cell inclusions with an affinity for neutral red, termed *NR bodies*, were found in the gonad wall of nearly every specimen of *Culex pipiens*, but were not found in *C. territans*. They resemble the inclusion bodies of certain viruses and at times closely simulate masses of rickettsiae, but are apparently distinct from the species here described. Their nature is unknown but their appearance and behaviour are consistent with their being living agents or the manifestations thereof. A degenerative process, in which NR bodies replace primary ovarian follicles in whole or in part, is described.

Inoculation and cultivation experiments resulted negatively for both rickettsiae and NR bodies.

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EXPLANATION OF PLATES XIII–XVII

PLATE XIII

Figs. 1–8. Smears illustrating variety of forms and staining intensities of rickettsia of *Culex pipiens*. Ovaries, hibernating females, Giemsa, × 1000.

A probable sequence in the development of certain forms is as follows: Small, densely staining coccoids increase in size without dividing (Fig. 3) and attain the size illustrated in Figs. 2–4, 7 and 8 ("intermediate" coccoids), while others become the "large" coccoids shown in Figs. 5 and 6. Within such enlarged forms, as in a matrix, are formed "daughter" individuals variable in number, size, shape and staining, which as a group retain the general outline of the "parent" body even after they are clearly to be resolved as individuals. Whole fields may be composed of groups the size and outline of which approach those of the accompanying enlarged coccoids (Figs. 4 and 5). The curved and bent rods, so characteristic of this rickettsia, are interpreted as "daughter" forms which have become released and are straightening out. See drawings, Text-fig. A.

Fig. 1. "Typical rickettsia" forms, i.e. small rods and coccoids, with paired forms predominating; enlarged coccoids intermingled.

Fig. 2. Irregular coccoids, unequal pairs, a few curved rods; nearly all forms except large coccoids represented.

Fig. 3. Coccoids of various sizes predominating.

Fig. 4. The rickettsiae of a single cell; a mixture of all forms.

Fig. 5. Rods and coccoid forms of all sizes, irregular as to outline and internal consistency, including some imperfect ring forms. The less densely stained portions of enlarged coccoids may in many cases be resolved into granules and rods, or both, illustrating the formation of "daughter" groups.

Figs. 6 and 7. Cell fragments showing an unusual proportion of enlarged coccoids. The lightly stained material consists of closely packed, smaller rickettsiae.

Fig. 8. Cell fragment; a mixture of various forms.

PLATE XIV

Gonads of *Culex pipiens*, neutral red preparations. NR bodies alone are stained, while rickettsiae, germ cells and pathological cells have the same appearance as in ordinary fresh preparations.

Fig. 9. Whole gonad, second instar larva. One NR body. ×220.

Fig. 10. Whole gonad, third instar larva. ×1000. Germ cells with cytoplasm filled with rickettsiae. One NR body (*NR*) at periphery. This gonad had a number of NR bodies scattered peripherally.

Fig. 11. Ovarian follicle, pupa. ×1000. One peripheral NR body (*NR*). The large, spherical, granular mass which exhibited no trace of stain, may be either a "pathological" germ cell packed with rickettsiae, or more probably an NR body which has lost its affinity for the stain.

Fig. 12. Ovarian follicle, pupa. ×1000. Loose mass of rickettsiae in "pathological" germ cell (*PC*). Other germ cells (*GC*) refractive, their rickettsiae barely discernible. The single NR body (*NR*) which was homogeneous and refractive when photographed, later faded and became granular.

Fig. 13. Gonad, third instar larva; fresh preparation of tissue teased in neutral red. ×1000. Two germ cells (*GC*), rickettsiae in cytoplasm appearing indistinctly granular, nuclei indicated by lighter areas. A granular NR body (*NR*) which was at first a deep red but had faded somewhat when photographed. The three adjacent bodies, which showed no trace of stain, are solid masses of granules and may be pathological germ cells but are probably NR bodies of which the matrix has lost its affinity for neutral red. This hanging-drop preparation was later fixed and stained with Giemsa and the same cells were found, undisturbed as to their relative positions. The NR body and its three companions all stained as reddish masses of closely packed granules, while the other cells stained as typical germ cells, with densely stained nuclei and blue cytoplasm unevenly filled with rickettsiae.

Fig. 14. Testis, adult. ×1000. A pathological cell (*PC*) consisting of a mass of rickettsiae suspended in colourless liquid. Neighbouring chambers are filled with normal germ cells which are also infected, but both rickettsiae and nuclei are invisible on account of refractivity of the cells. The septa contain NR bodies (*NR*) of various sizes.

PLATE XV

Figs. 15–20. Giemsa-stained sections of testis of fourth instar larvae, showing germ cells infected with rickettsiae, pathological cells and NR bodies. The tissue surrounding the testis is fat body. ×1000.

Fig. 15. Several pathological cells (*PC*), nuclei not recognizable, filled with densely stained rickettsiae. Coccoids and rods, some surrounded by haloes, in cytoplasm of normal germ cells, which exhibit varying degrees of infection. No NR bodies in this field.

Fig. 16. Two NR bodies (*NR*) in tip of anterior end, appearing as loose masses of granules. Several pathological cells (*PC*). The NR bodies and their granules in Giemsa sections usually stain bluish while the rickettsiae, in pathological cells or elsewhere, are reddish.

Fig. 17. Two large, densely stained pathological cells (*PC*). No NR bodies.

Fig. 18. Large pathological cell (*PC*); another with pycnotic nucleus. One small, dense, granular NR body (*NR*) in testis wall.

Fig. 19. Large, loosely granular NR body (*NR*) projecting inward from testis wall; outline of enclosing cell seen at upper side of NR body. Germ cells with varying degrees of infection.

Fig. 20. End products of pathological cells (*PC*); an extreme example in which a chamber at the posterior end of the testis contains no sperms but is filled with pathological cells. They may become large, irregular, poorly defined masses containing fragments of nuclear material and rickettsiae.

Fig. 21. Testis, adult; fresh preparation, neutral red. ×1000. Sperm-filled chamber with residual mass usually present in *Culex pipiens*, which represents end product of pathological cells. There may be one or several of these masses. There are no NR bodies in this field though they were present in this testis in their usual position at the anterior end.

PLATE XVI

Figs. 22–27. Fresh preparations, neutral red, of gonads of *Culex pipiens* showing variations in morphology of NR bodies.

Fig. 22. Unbroken gonad, second instar larva. A granular NR body (*NR*) stained with neutral red. ×2000.

Fig. 23. Unbroken gonad, third instar larva. Two NR bodies (*NR*), both originally deep red, but faded to light pink when photographed. Both show typical, granular appearance, which increases in distinctness as the neutral red stain fades. ×2000.

Fig. 24. An NR body (*NR*) in follicle, adult unfed female. Photographed immediately after dissection, this shows characteristically intense staining. After standing several hours this had faded and become finely and evenly granular. ×1000.

Fig. 25. Ovarian follicle, pupa. "Double" NR body, light pink and finely granular. ×1000.

Fig. 26. Irregular NR bodies in gonad, fourth instar larva. ×1000.

Fig. 27. Group of small NR bodies in unbroken gonad, third instar larva. ×1000.

Figs. 28–35. Smears of pathological ovarian follicles, i.e. those replaced in whole or in part by NR bodies. Figs. 28, 29 and 30 show individual NR bodies. In Figs. 31–34 the granules are probably derived mostly from NR bodies since the latter formed the greater part of the material smeared. The similarity of NR body granules and rickettsiae in smears is very great.

Figs. 28 and 29. Two NR bodies. In smears they tend to retain their original shape and often give the appearance of having broken into fragments of matrix packed with granules, while germ cells rupture readily and permit their contents to flow out in a thin film. The granules of the NR bodies are rickettsia-like in morphology but consist chiefly of tiny coccoids and very short rods. Isolated individuals may stain faintly or intensely. ×1000.

Fig. 30. Single NR body; cohesive fragments of matrix with densely stained granules. Isolated granules are tiny coccoids or rods with occasional ring and curved forms. ×1000.

Fig. 31. Smear of pathological follicle. Masses of very minute coccoids, probably derived from NR bodies. ×1000.

Fig. 32. Smear of pathological follicle. Granules probably derived from NR bodies; a high proportion of single coccoids, some of them surrounded by sharply outlined clear areas or haloes, indicated by arrow. ×1000.

Fig. 33. Smear of pathological follicle. Granules of various sizes with preponderance of very minute and faintly stained forms, probably derived from NR bodies. ×1000.

Fig. 34. Smear of pathological follicle. Fragments of NR body with a number of isolated rickettsia-like forms, including minute rings and curved rods. ×1000.

Fig. 35. Smear of pathological follicle. Minute rickettsia-like forms; whether NR body granules or rickettsiae uncertain, though nucleus would indicate the latter.

PLATE XVII

Fig. 36. Ovary, hibernating female, fresh preparation, neutral red, showing scattered NR bodies and the pathological follicle (*PF*) shown in Fig. 37. ×110.

Fig. 37. Same preparation as Fig. 36 showing pathological follicle with NR bodies of various sizes and staining intensities. No normal germ cells recognizable. The large, irregular NR body at right is in tissue between ovarioles, where they may at times be numerous. ×1000.

Figs. 38 and 39. Sections of ovary of hibernating female which exhibited an exceptional number of NR bodies and pathological follicles. Fig. 38 shows a large, trilobed, granular NR body (*NR*) and a tiny spherical one beside it. The large oocyte is filled with rickettsiae (*R*) normal in appearance and staining intensity, while the rest of this follicle has been replaced by NR bodies (*NR*). Some of the latter are morulate, i.e. exhibit subdivisions into densely stained masses of uniform size. In Fig. 39, another section of the same follicle, these subdivisions are in turn seen to be composed of smaller granules. In the oocytes, haloes surrounding groups of rickettsiae are conspicuous. Regaud, iron haematoxylin. ×1000.

Fig. 40. Pathological follicle showing remains of oocyte with densely stained, reddish rickettsiae (*R*), the rest of the follicle occupied by NR bodies (*NR*). The latter stain greenish or bluish. While some are granular others are homogeneous and several of the latter contain vacuoles. Regaud, Giemsa. × 1000.

Fig. 41. Section of normal follicle, hibernating female, showing large oocyte, its cytoplasm packed with faintly stained rickettsiae, together with a few large, densely stained forms which may represent exceptional forms of the rickettsia or some other organism. The groups of granules in the follicular epithelium are nuclei. Zenker, iron haematoxylin. × 1000.

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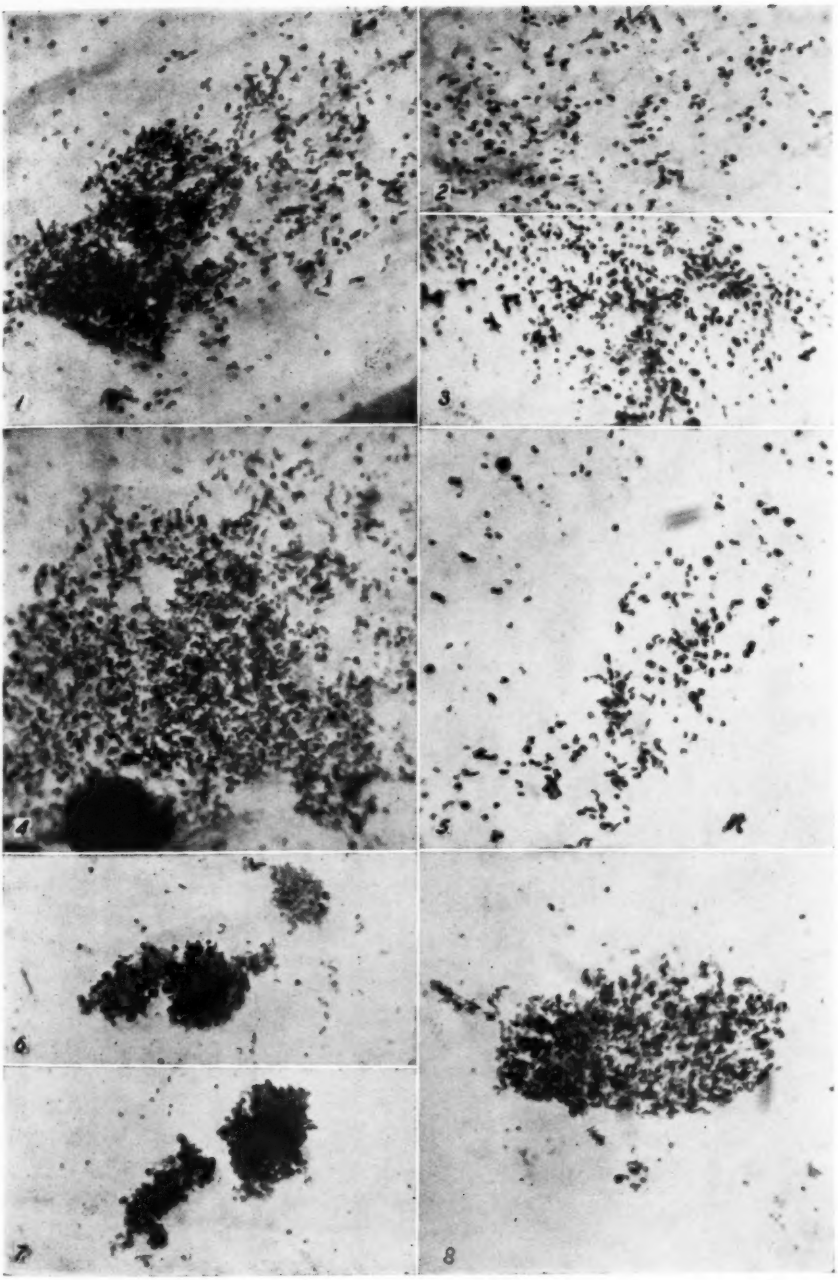
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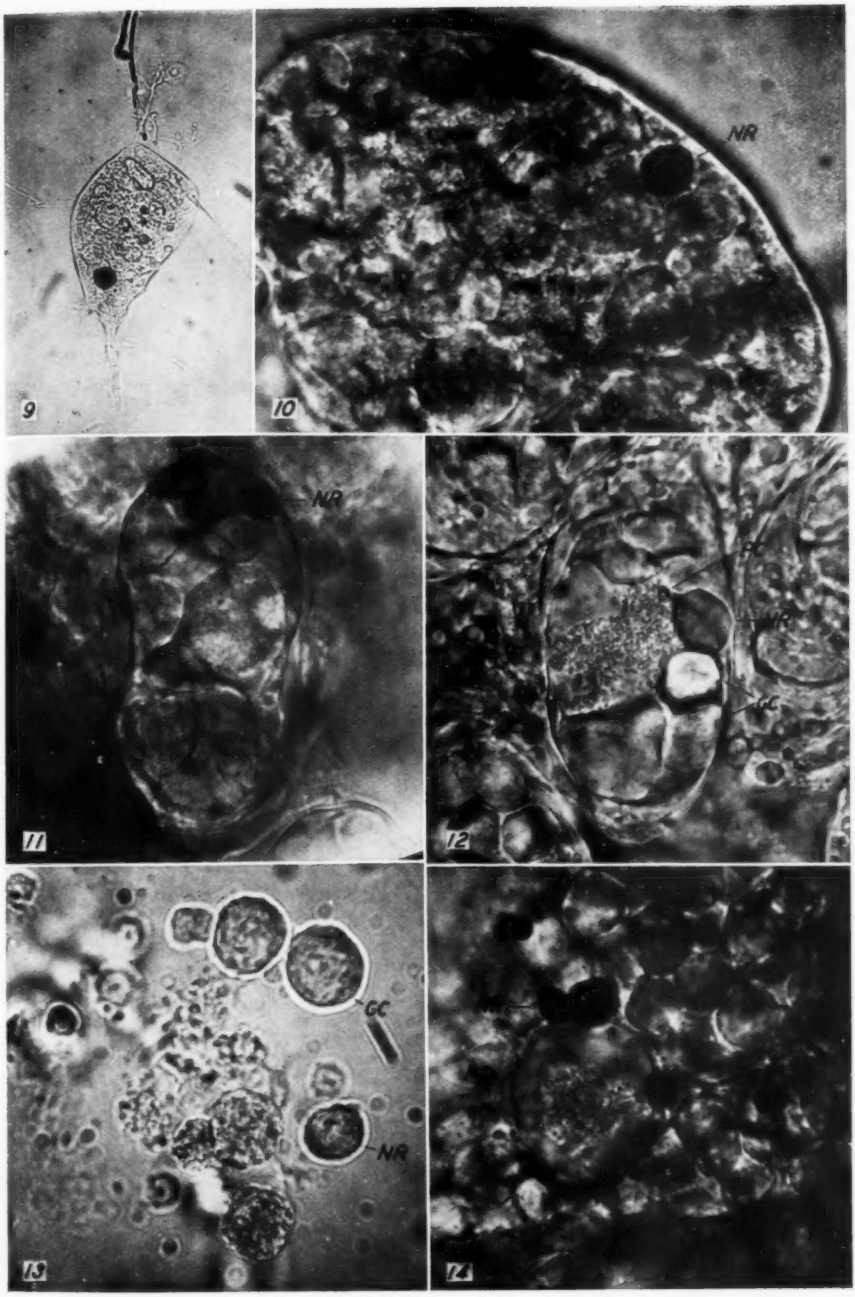
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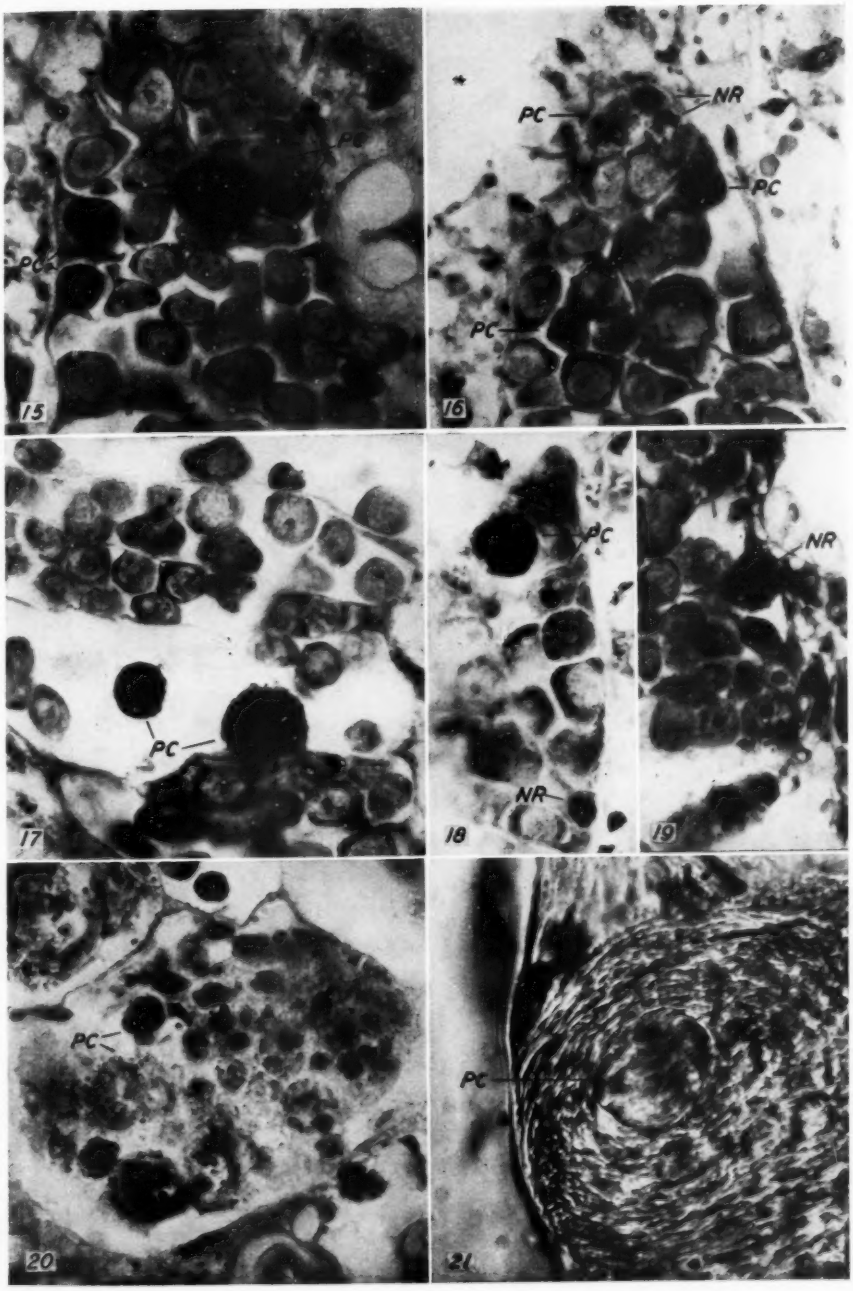
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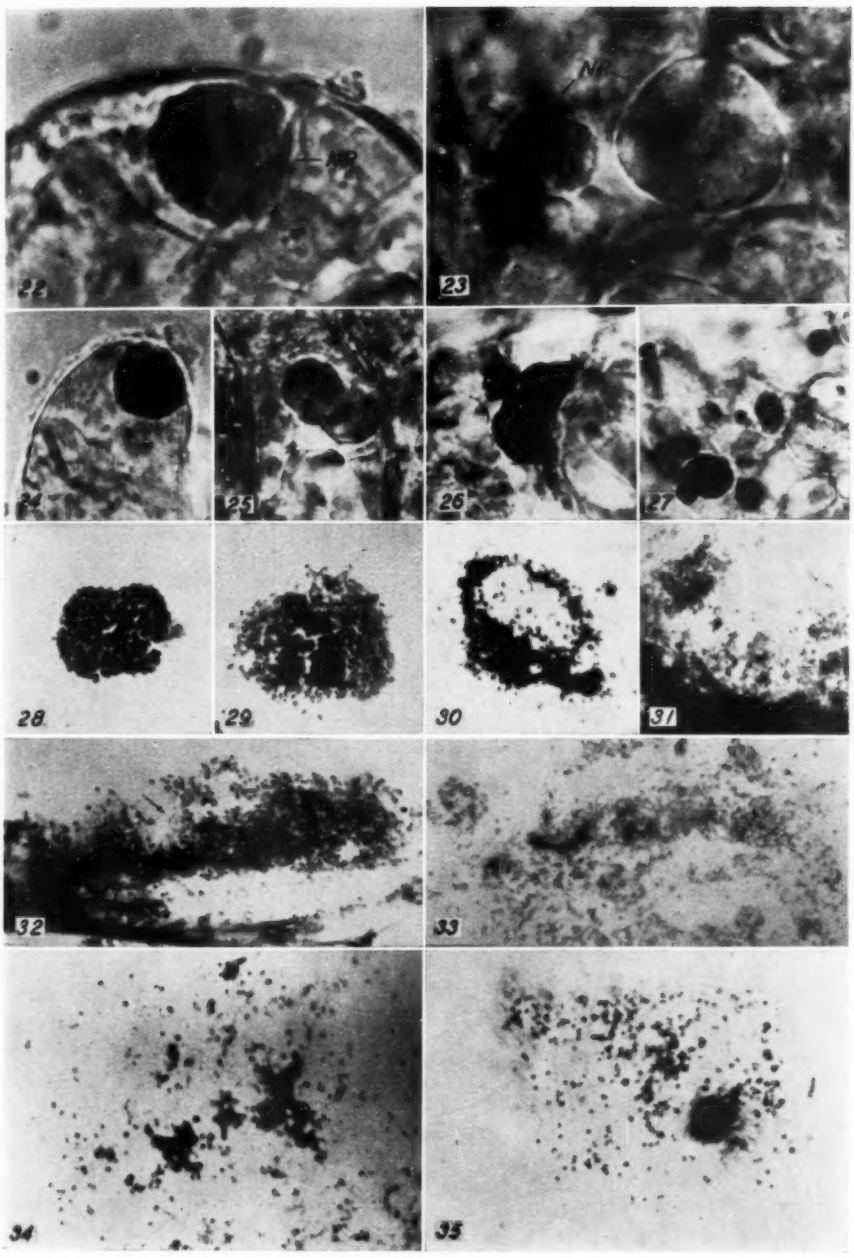
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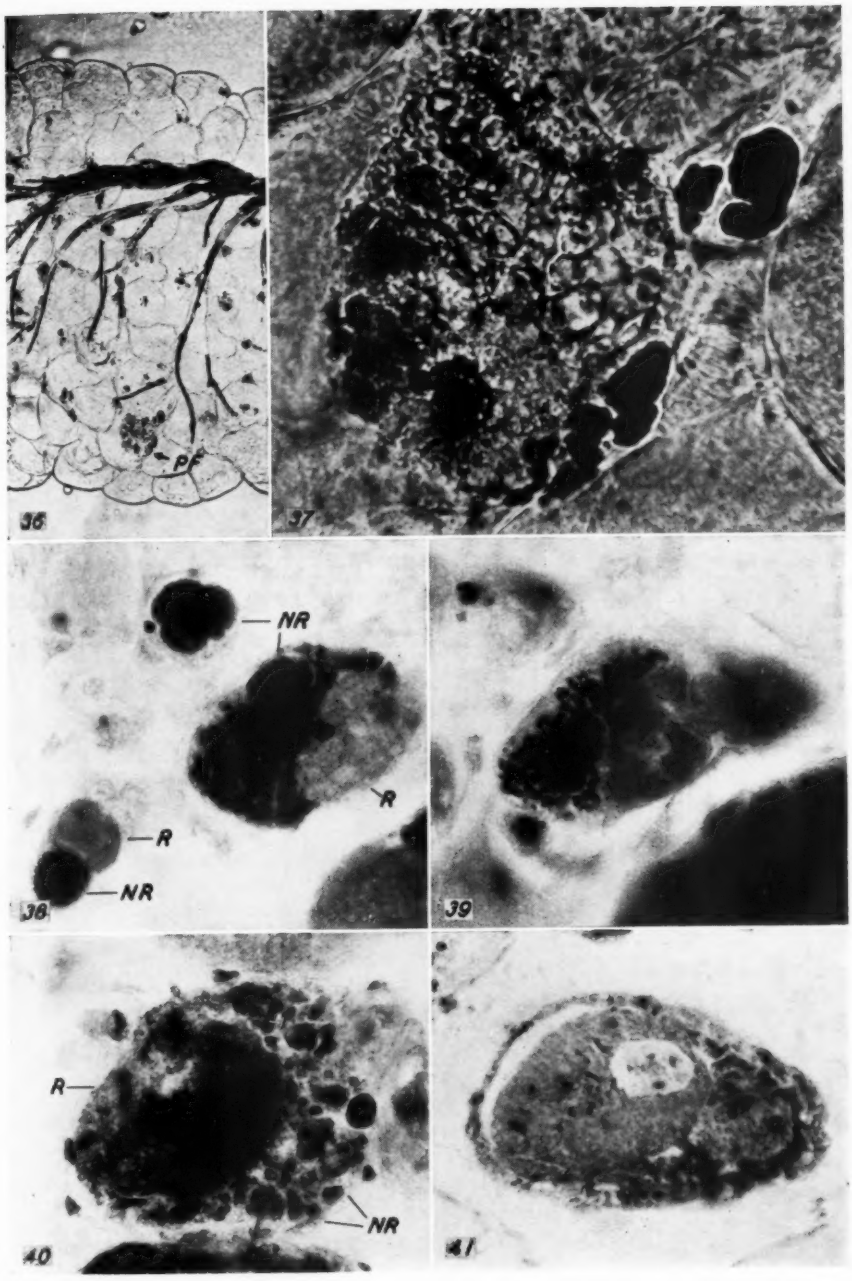
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NOTES ON NEW ASPIDOGASTRID SPECIES, WITH A CONSIDERATION OF THE PHYLOGENY OF THE GROUP[1]

By Prof. ERNEST CARROLL FAUST, M.A., Ph.D.
and CHUNG-CHANG TANG

(With 14 Figures in the Text)

Introduction

The group of trematodes to which *Aspidogaster* and its relatives belong is one of the most instructive in the phylum Platyhelminthes, yet scant attention has been paid to these species by students of helminthology. Since the discovery of *Aspidogaster conchicola* by von Baer in 1826 the following genera have been described: *Aspidogaster* von Baer, 1826; *Cotylaspis* Leidy, 1857; *Macraspis* Olsson, 1868 (*vel* 1869); *Stichocotyle* Cunningham, 1884; *Platyaspis* Monticelli, 1892; *Cotylogaster* Monticelli, 1892; *Lophotaspis* Looss, 1901; and *Lobatostoma* Eckmann, 1932. One or more species of each of these genera have been described from fresh-water hosts in Europe, North America, Africa, Asia Minor, the U.S.S.R. and China, and from marine hosts in the Mediterranean area, the Firth of Forth, the North Sea, the Atlantic coast of North America, Ceylon and Western Australia. The hosts are cold-blooded animals and include gasteropods, bivalves, crustaceans, fishes and turtles. In a previous communication (Faust, 1922) one of us reported *Aspidogaster conchicola* from *Unio* sp., *Vivipara lapillorum*, *V. catayensis*, *Leuciscus aethiops* and *Amyda sinensis* in China. The present study permits us to add a new species each of *Lophotaspis* and of *Cotylaspis* from the intestine of *Amyda tuberculata*, from Fukien Province, China, and a new species of *Stichocotyle* from the spiral valve of the cow-nosed ray, *Rhinoptera quadriloba*, taken from Biloxi Bay, Mississippi. These species will now be described in detail.

(1) **Cotylaspis sinensis** nov.spec.

(Fig. 1)

Host: *Amyda tuberculata*, small intestine.

Habitat: Foochow, Fukien Province, China.

Date collected: April, 1935.

Three specimens of this species were collected from the small intestine of one of the same fresh-water turtles from which *Lophotaspis orientalis* was obtained, and three other specimens were discovered in other turtles of the same species examined at the same time. Superficially this fluke (Fig. 1)

[1] Contribution from the Parasitology Laboratory, Department of Tropical Medicine, Tulane University of Louisiana, New Orleans, La., and from the Fukien Science Institute, Foochow, China.

resembles *Cotylaspis insignis* Leidy, 1857, although the disc portion is distinctly broader. In hot formalin-fixed, carmine-stained *toto* mounts the over-all length of the worm averages 1·5 mm., the length of the ventral sucking disc is 1·0 mm., and the greatest breadth is 1·16 mm. The integument is relatively thin and is aspinose. The sucking alveoli of the disc (Fig. 1) consist of an

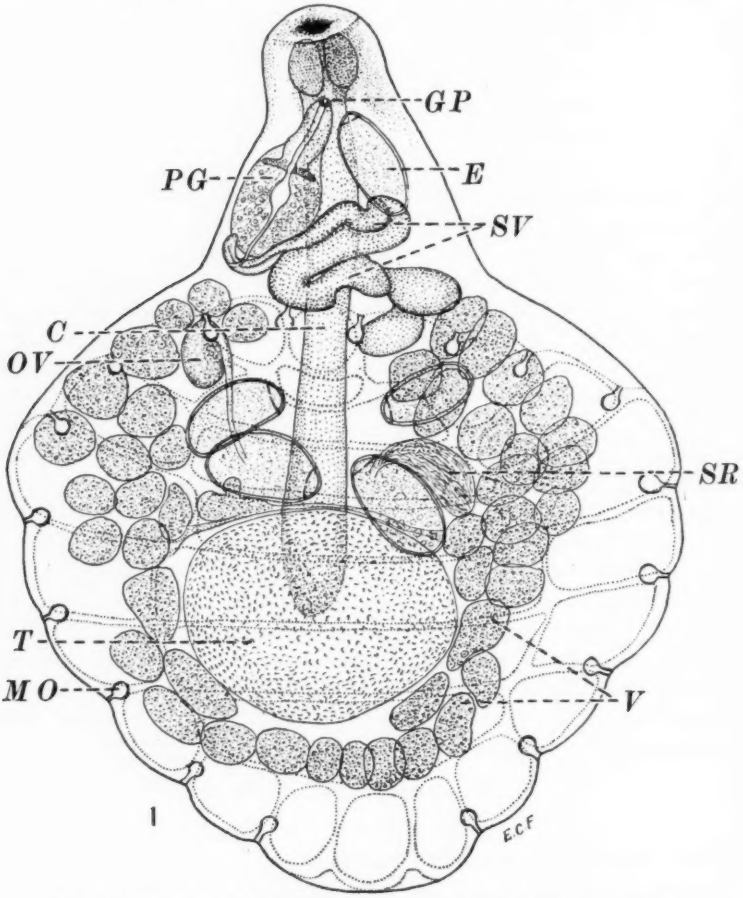

Fig. 1. Dorsal view of *Cotylaspis sinensis*, showing the important externa and internal structures. ×87.

outer rim of 20 and a central row of 7, making a total of 27, as contrasted with 18 and 7 respectively in *C. lenoiri* Poirier 1886, typically 20 and 9 in *C. insignis*, 22 and 10 for *C. cokeri* Barker & Parsons, 1914, and 34 and 16 for *C. stunkardi* Rumbold, 1927. The acetabular structure conforms more closely to *C. insignis* than to the other described species of *Cotylaspis*. At

the margin of each septum separating adjacent marginal alveoli there is a conspicuous bottle-shaped marginal organ (*MO*).

As in other species of the genus, the mouth of *C. orientalis* is situated on a truncated conical prominence. Almost immediately inside the oral opening is the relatively small pharynx, which measures about 0·1 mm. in length by 0·12 mm. in transverse diameter. Internal to the pharynx is a simple sacculate caecum (*C*) which ends blindly somewhere near the mid-plane of the testis. The testis (*T*) is a comparatively huge organ, transversely ovoid in contour, situated in the posterior half of the worm, slightly to the left of the mid-line. It measures 0·38 by 0·47 mm. In the three *toto* mounts available for study the vas efferens has not been found, but the contorted swollen seminal vesicle (*SV*) twists on itself three times before constricting into a much smaller tubule as it coils tightly on itself before entering the conspicuous cirrus sac. This latter organ is filled almost completely in its posterior two-thirds by the prostate glands (*PG*). In the anterior third the male tubule passes through a semi-glandular septum into a rather thick-walled, semi-muscular portion of the sac, the ejaculatory duct. No cirrus organ has been observed. Except for the excessive size of the testis and of the seminal vesicle (the latter being an apparent adaptation to the former), the male genitalia closely conform to those of *C. insignis*. The genital pore is situated ventrally in the mid-line, just behind the pharynx. The vitellaria (*V*) are large discrete follicles of ovoid or polygonal contour, disposed in two to three rows on both sides of the disc portion of the body and uniting in a single row posterior to the testis. The transverse vitelline ducts proceed mesad just anterior to the testis. On the left side there is a conspicuous vitelline reservoir. The minute retort-shaped ovary (120μ in length by 86μ in cross-section) is situated far anteriorly in the upper left quadrant of the disc. The proximal portion of the oviduct has been traced down to the region of the vitelline reservoir but its distal end has not been seen. Nor has the ootype been found. However, a large seminal receptacle (*SR*) has been located to the right and anterior to the testis.This observation contrasts with that of Osborn (1904), who states (p. 228) that "there is no seminal receptacle in *C. insignis*". The outline of the uterus is indistinct, but this organ probably consists of only a single S-shaped tubule and contains at most five large canary yellow, fairly thin-shelled eggs, with a distinct operculum and having a measurement of 168–182 by 98–112μ, with an average of 177 by 106μ. These eggs are in an early stage of embryonation.

The excretory system has not been observed.

C. sinensis is specifically distinguished on the basis of the number of alveoli of the sucking disc, the large size of the testis, seminal vesicle and vitellaria, the presence of a large seminal receptacle, the short uterus and the small number of eggs.

Types and paratypes have been deposited in the Faust Helminthological Collection.

(2) **Lophotaspis orientalis** nov.spec.

(Figs. 2–5)

Host: *Amyda tuberculata*, stomach and small intestine.

Habitat: Foochow, Fukien Province, China.

Dates collected: 1933, 1934, 1935.

This fluke was recovered in small numbers on four separate occasions in an examination of numerous specimens of the common fresh-water turtle in the vicinity of Foochow, China. The living worms were pinkish in colour and were seen with difficulty as they adhered to the wall of the stomach and duodenum. The worms were fixed under slight pressure in steaming 4 per cent. formalin, were later stained with a modified borax-carmine preparation and were mounted *in toto* for study. The adult worms (Fig. 2) are elongated oval, more or less slipper-shaped, with an attenuated anterior end and a gracefully rounded posterior end. The fixed specimens average 6 mm. in length by 2·3 mm. in breadth, while the ventral sucking disc, symmetrically oval in contour, measures 3·7 mm. in length by 1·8 mm. in breadth. As far as could be discovered, the body is aspinose. The alveoli (*AL*) of the disc are irregularly oval, with a tendency to hexagonal contour. Their greater diameter is in the transverse plane. These alveoli are arranged in four rows, a characteristic of the genus, there being a total of 54 separate alveoli. In the midst of the triangle of muscle bands between each three alveoli there is a finger-like papilla (*P*), which is either partially introverted or considerably everted. Regularly six of these papillae can be found around each of the alveoli of the middle two rows and three papillae on the median aspect of each alveolus of the outer rows. Rarely only five of these papillae have been found around the alveoli of the middle rows. There is a total of about 65 of these papillae. Marginal organs (*MO*), apparently having a secretory function, are situated on the edge of the disc, midway between each two outer alveoli. The anterior portion of the disc is shown conventionally in Fig. 3.

The oral opening of this worm has the shape of a morning-glory calyx and is moderately muscular. It leads through a short prepharynx into a large muscular pharynx (0·48 by 0·64 mm.), internal to which there is a long unbranched sacculate caecum (*C*), which becomes smaller in diameter as it proceeds to a blind end near the posterior margin of the testis. The single testis (*T*) lies in the distal fourth of the worm, somewhat to the left of the mid-plane. It is broadly oval (0·86 by 0·77 mm.). From its anterior aspect there arises a vas deferens (*VD*), which terminates at the genital pore (*GP*), in a median position just anterior to the cephalic margin of the pharynx. Prostate glands are apparently lacking and no distinctly differentiated cirrus organ has been seen. The ovary (*O*) lies tangentially to the upper right of the testis. It is subspherical in shape, measuring about 0·5 mm. in the longitudinal plane by 0·45 mm. in transverse diameter. The oviduct (Fig. 4, *OD*) arises from the anterior ventral aspect of the ovary and descends to the ootype (*OO*)

as a short, slightly curved tubule. The vitellaria (*V*) consist of groups of discrete glands overlying the outer series of alveoli and extending from the second transverse row of alveoli to the antepenultimate row. In this latter

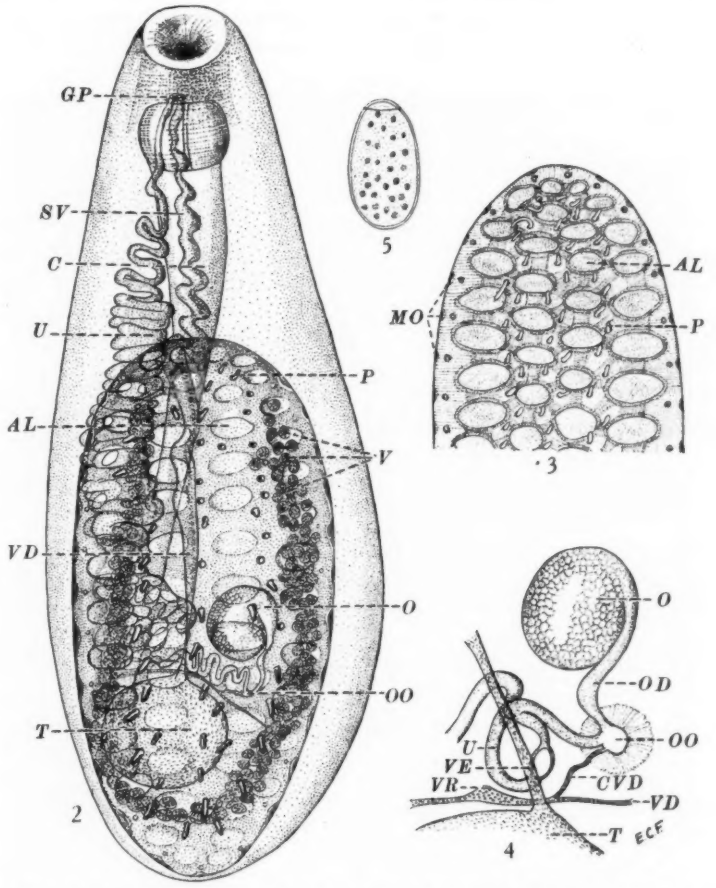

Fig. 2. Dorsal view of *Lophotaspis orientalis*, showing important external and internal structures. × 22.

Fig. 3. Ventral view of anterior portion of ventral sucking disc of *Lophotaspis orientalis*, showing relationship of alveoli, papillae and marginal organs. Somewhat conventionalised. × 22.

Fig. 4. Details of the genitalia in the region of the ootype of *Lophotaspis orientalis*, dorsal view. 36.

Fig. 5. Uterine egg of *Lophotaspis orientalis*. × 145.

position the two lateral groups turn mesad and unite in the mid-line. The two transverse vitelline ducts (*VD*) proceed from points just anterior to the proximal border of the testis and unite at a point slightly to the left and

posterior to the ootype.[1] Here the common duct (*CVD*) proceeds into the ootype. Somewhat to the left of the junction of the transverse ducts there is an expansion (*VR*) which probably is a vitelline reservoir. The ootype (*OO*) is surrounded by a spherical cluster of Mehlis' glands. The uterus (*U*) emerges from the ootype to the left between the oviduct and the common vitelline duct, proceeding first transversely as a convoluted tubule, but when it arrives on the left of the vas efferens continues cephalad as a highly swollen convoluted tube to the genital pore. Only in the anterior third of the body does the tube tend to straighten out and become more attenuate. Eggs may accumulate anywhere within the uterus but are most numerous at the base of its longitudinal course. These eggs (Fig. 5) are typically ovoid in shape, slightly more rounded posteriorly than anteriorly, have a golden yellowish tinge, a moderately thin shell with an operculum, and no especial thickening of the shell at the posterior end. All eggs studied were in the early stages of cleavage. They measured 124–135μ in length by 48–69μ in breadth with an average of 130 by 56μ.

The excretory system in this species was not studied.

This organism conforms to the criteria established for the genus *Lophotaspis*. Specifically it differs from the previously described species (*L. vallei* [Stossich, 1899], *L. margaritiferae* [Shipley & Hornell, 1904], *L. macdonaldi* [Monticelli, 1892] and *L. interiora* Ward & Hopkins, 1931), in its size, the relative extent of the ventral disc, the number of papillae between the alveoli, the diameter of the vas efferens and the seminal vesicle. Even without considering the geographical location and the species of host where this trematode was found, there is abundant evidence warranting its creation as a distinct species.

Type and paratypes have been deposited in the Faust Helminthological Collection.

(3) **Stichocotyle cristata** nov.spec.

(Figs. 6–14)

Host: *Rhinoptera quadriloba*, spiral valve.

Habitat: Biloxi Bay, Mississippi.

Date collected: October, 1932.

A single specimen of this unique form was collected and sent to the writer for identification by Mr Stewart Springer, Director of the Caribbean Biological Laboratory, Biloxi, Mississippi. No information is available regarding its shape or colour as a living organism, but it is not unlikely that the coiled condition of the preserved specimen (Fig. 6) was fairly characteristic of its natural state. While the living worm was probably pink or salmon-coloured, the preserved worm was creamy white. The entire ventral side was found to

[1] Recently one of us (C.-C. T.) has observed in living specimens a Laurer's canal, filled with spermatozoa, arising from the region of the ootype and opening on the dorsal surface in approximately the same plane.

be provided with a complicated acetabular structure extending from a position somewhat behind the genital pore to near the caudal extremity. The worm when stretched out measured 7·9 cm. in length, with a greatest breadth of about 2·0 mm. and a greatest thickness (inclusive of acetabula) of about 2·4 mm. The integument was aspinose. The most conspicuous external features were (1) the thick-walled, funnel-shaped mouth cavity, (2) the conspicuous

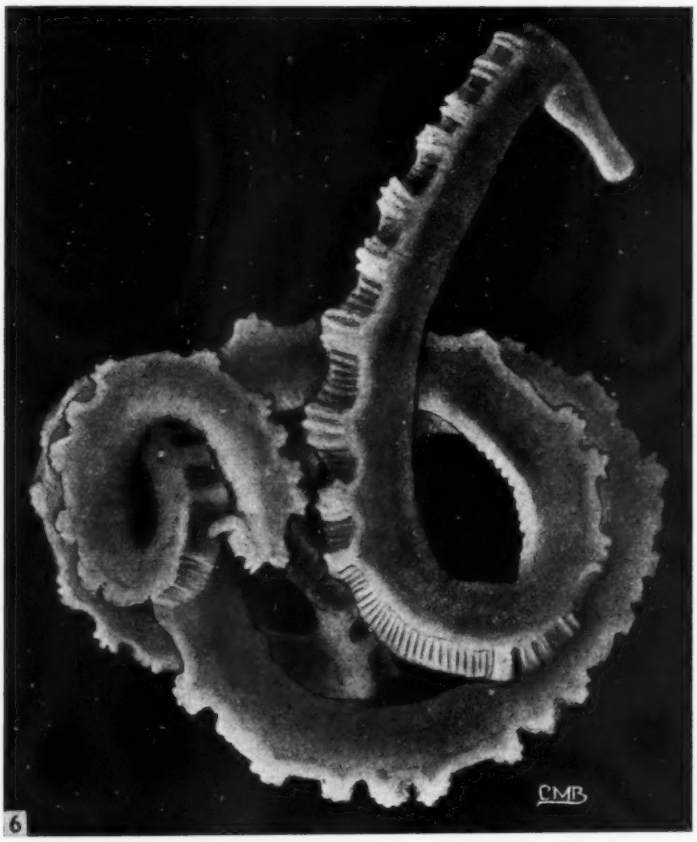

Fig. 6. Camera lucida sketch of *Stichocotyle cristata*, showing dorsal view of head (at upper right), ventral and lateral views along the acetabular apparatus and caudal extremity (at termination of the coil on the left side of the sketch). ×8.

slightly curved cirrus organ situated medially just a short distance behind the oral sucker (Fig. 7), (3) the acetabular apparatus (Figs. 7, 8, 9), and (4) the conoidal distal end of the worm (Fig. 9), directed almost at right angles to the longitudinal axis of the body. Likewise, there was a distinct sinusate blackish line seen in lateral view on either side of the worm where the aceta-

bular structure appeared to join the body. After the worm had been prepared as a *toto* mount this line was identified as the vitelline follicles of that side.

The acetabular structure required particular study. It was found to consist of two portions, (1) a short anterior sucking disc (Fig. 7) about 3 mm. long, divided into eight sucking cups by seven transverse septa, and (2) a long acetabular complex, consisting of seventy-nine elevated lug-like crests, each typically provided with three transverse ridges. The crests were separated from one another by trough-like portions of considerably greater length, the latter being also divided by numerous transverse septa. This complicated sucking apparatus has thus been found to conform fundamentally to the definition of the genus *Stichocotyle*, which has a single long row of sucking cups, although it is modified and elaborated greatly when compared with the type species, *S. nephropis* Cunningham, 1894.

After camera lucida habit sketches of the worm (Fig. 6) and of its several unique external features (Figs. 7–9) had been prepared, the worm was carefully untangled and flattened between two slides, then stained in a borax-carmine solution, dehydrated, cleared and mounted *in toto* on its side. A study was then made of the internal anatomy of the worm. The cup-shaped mouth cavity (Fig. 10) was found to lead directly into the dense muscular pharynx (about 0·8 mm. deep and 1·0 mm. in transverse section). Behind the pharynx the long sacculate intestinal cecum (*C*) proceeds through the entire length of the worm (Figs. 11, 12), ending blindly in the extreme caudal tip just in front of the excretory bladder. The excretory bladder (*EB*) is a minute muscular organ situated just caudally to the posterior tip of the intestine. From it there arises a single delicate median tubule, which proceeds anteriad to the position where the caudal portion of the body joins the main trunk. Here it bifurcates and each main collecting tubule proceeds anteriorward, dilating considerably *en route*. These tubules have been traced up to the region of the pharynx, but secondary or collateral tubules have not been observed.

The primary male organ consists of a single greatly elongated ovoid testis (Fig. 12, *T*), which is slightly twisted on itself in its anterior third. This testis lies approximately halfway between the anterior and posterior ends of the worm, between the 28th and 30th acetabular crests. The proximal end of the vas efferens has not been discovered but the distal end, some distance before the duct joins the seminal vesicle, is dilated and moderately conspicuous (Fig. 10). As this duct enters the cirrus sac it becomes swollen and highly convoluted to form the seminal vesicle (*SV*). While yet within this sac it again becomes attenuated and passes through the region of the prostate glands (*PG*), and then proceeds as an ejaculatory duct up to the genital pore (*GP*). It terminates in a very distinct thick-walled muscular cirrus or penial organ (*CI*). The ovary (Fig. 11, *O*) is located far anterior to the testis. It is situated at the level of the eleventh acetabular crest, between the first and second seventh of the fluke's body. It has more or less the shape of an inverted retort and measures about 0·568 mm. in transverse diameter by 0·773 mm. in its

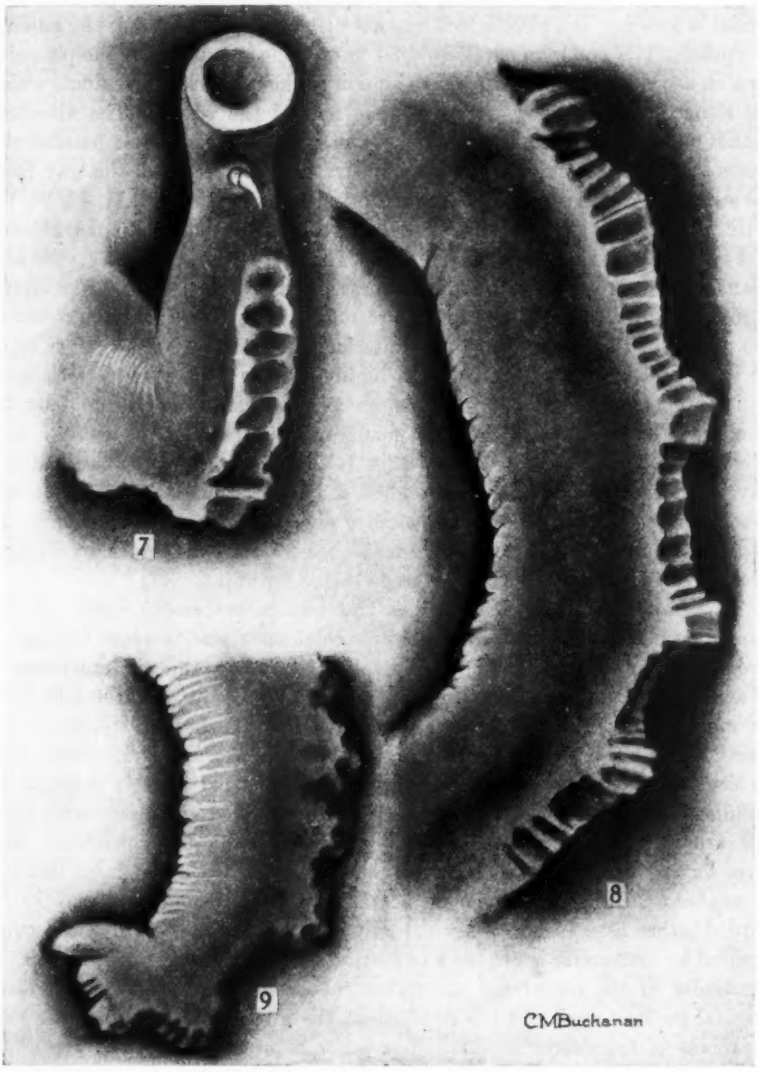

Fig. 7. Head end of *Stichocotyle cristata* (ventral view), showing oral opening, cirrus organ and anterior compound acetabulum. ×17.

Fig. 8. Lateral view of middle portion of *Stichocotyle cristata* in vicinity of the testis, showing body curvature and acetabular crests and troughs. ×17.

Fig. 9. Posterior extremity of *Stichocotyle cristata* (lateral view), showing acetabular crests and troughs, the line of the vitelline follicles and the abrupt angle at the caudal tip. ×17.

greatest diameter (which is oblique). The oviduct descends ventrally into the ootype (*OO*). Midway between the ovary and the ootype, but somewhat mesial in location, is a mass which was not clearly seen but may be the seminal receptacle. The common vitelline duct enters the ootype from the posterior median aspect. It is formed from the union of the two transverse ducts which are situated only a very short distance behind the ootype. The vitellaria consist of a right and a left column of discrete follicles which parallel the crests and troughs of the acetabular apparatus and extend all the way from the second acetabular crest down to the penultimate crest (Figs. 11, 12, 13, *V*). Within the ootype (Fig. 11, *OO*) several eggs can be seen in process of synthesis. They are situated in an **S**-shaped canal which traverses Mehlis' glands and opens on the meso-posterior margin of these glands, to continue as the proximal limb of the uterus (*U*). This tubule descends in wide serpentine coils down to the middle of the body, where, just anterior to the testis (Fig. 12, *U*) it turns on itself and proceeds anteriad as a highly swollen tube packed with eggs. As it approaches its outer termination its coils become wider and its diameter smaller. Finally it parallels the ejaculatory duct up to the genital pore (Fig. 10, *U*). The eggs of *S. cristata* (Fig. 14) are regularly elongate-ovoid and have a relative thick, golden yellow shell, which has a distinct operculum and a teat-like thickening at the abopercular end. All uterine eggs were in an early stage of embryonation. They measured 97–109μ in length by 57–65μ in transverse diameter, with an average of 103 by 62μ.

In comparison with *S. nephropis* the species herein described exhibits numerous minor differences but most conspicuously stands apart because of two prominent characters, (1) the acetabular structure and (2) the presence of a single testis instead of two. In *S. nephropis* the multiple acetabula (*vide* Odhner, 1910) are distinctly separated from one another by spaces several times their own breadth. Although stretched more or less transversely into lenticular cups across the ventral side of the worm, particularly through the middle two-thirds of the body, they are never provided with transverse septa nor are they ever fused. *S. cristata* may be reasonably considered as a form having acetabula which have been elaborately modified from those of *S. nephropis*. Its anterior ventral acetabulum is compound and may be regarded either as a fusion of several cups, or a single one which has become divided by transverse septa, or a combination of these two processes. In the remainder of the acetabular apparatus the crests most probably represent original sucking cups and the depressions the interspaces. Certain it is that a process of transverse septation has become inpressed on both the crests and interspaces. The single enormous testis in *S. cristata* may possibly be a fusion of two smaller testes such as are present in *S. nephropis*, but it seems more likely that it represents a more primitive condition, such as is exemplified by all described species of aspidogastrids except *S. nephropis*.

While it seems desirable to leave *S. cristata* in the genus *Stichocotyle*, its significant deviations from type require an emendation of the generic concept

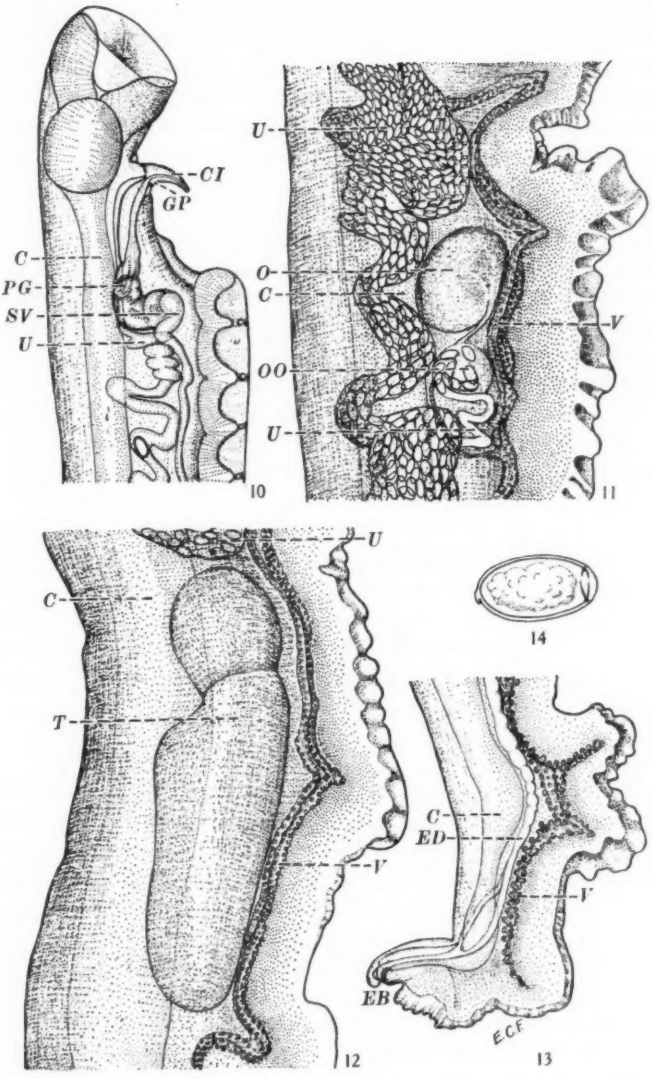

Fig. 10. Anterior end of body of *Stichocotyle cristata* (lateral view), showing internal organs and portion of anterior compound acetabulum. ×22.

Fig. 11. Segment of *Stichocotyle cristata* (lateral view), showing important organs in the vicinity of the ootype. ×22.

Fig. 12. Segment of *Stichocotyle cristata* (lateral view), showing important organs in the vicinity of the testis. ×22.

Fig. 13. Distal end of *Stichocotyle cristata* (lateral view), showing organs in this portion of the worm. ×22.

Fig. 14. Uterine egg of *Stichocotyle cristata*. ×175.

to include (1) the modified acetabular structure and (2) the single testis. These differences which exist in the only two known species of *Stichocotyle* may be appropriately distinguished by considering these two species as belonging to two separate subgenera, of which the type subgenus *Stichocotyle* is represented by the type species for the genus, *S. nephropis*, while for the new species, *S. cristata*, a new subgenus, *Multicalyx*, may be erected.

Genus **Stichocotyle** Cunningham, 1884, emend.

Aspidogastrid species having a single row of acetabular cups, which may either be simple and separated from one another (as in *S. nephropis*) or may be subdivided by transverse septa, which may not only divide the original acetabula into several pockets but also similarly divide the interacetabular spaces (as in *S. cristata*); with one or two testes.

Subgenus **Stichocotyle** nov.

With simple acetabula, separated by interspaces; testes two.

Subgenus **Multicalyx** nov.

With acetabula subdivided by transverse septa, which also produce transverse divisions of the interacetabular spaces on the ventral side of the body; testis single.

Monotypic. Represented by *Stichocotyle* (*Multicalyx*) *cristata* nov.spec.

The holotype specimen of this worm has been deposited in the Faust Helminthological Collection.

Discussion

The systematic position of the group, to which the three new species, *Cotylaspis sinensis*, *Lophotaspis orientalis* and *Stichocotyle cristata*, belong, has for many years been in doubt. Considered by some workers as belonging to the Monogenea, by others to the Digenea, and yet by other investigators as members of an independent rank intermediate in position between the Monogenea and the Digenea, no definite decision has ever been made. Assuredly they are not members of the Monogenea: they lack posterior sucking discs and chitinous hooklets or anchors; the excretory pore or pores are posterior in position rather than anterior, and their intestinal tract is always rhabdocele in type. On the other hand, there is no evidence that they have an alternation of generations, a requisite for inclusion in the Digenea. The fully developed embryo within the egg-shell, which breaks through the opercular opening to proceed with its development, is a larva already possessing the fundamental characteristics of an adult, such as the ventral sucking disc, the excretory system with a posterior opening and the rhabdocele gut (for *Cotylaspis insignis* see Osborn, 1904; for *Aspidogaster conchicola* see Faust, 1922). This immature worm usually lacks any suggestion of a ciliated epithelium, although Fuhrmann (1928) states that anterior and posterior groups of cilia are present in this

stage of *Cotylogaster* and Manter (1932) described three ciliated plates for the larva of *Lophotaspis vallei*.

Although the complete life cycle of members of this group is unknown, there is reason to believe that its development is fundamentally direct, *i.e.* this young worm develops directly into an adult, either in the same host or in another individual of the same or different species. The simplest type of host relationship is obviously that in which a mollusc, either a gasteropod or a bivalve, is the sole host to the worm, such as appears to be the case most usually with *Aspidogaster conchicola*, obtained on numerous occasions from the lymph sinuses, hermaphroditic gland or renal organ of gasteropods or in the mantle cavity, pericardial or renal cavity of the Unionidae. When such infected hosts are ingested by cold-blooded vertebrates, such as fishes, frogs and reptiles, the parasite has frequently demonstrated its ability to withstand digestion and to attach itself to the wall of the stomach or small intestine of this vertebrate, thus initiating a second host association. A more complicated condition is present in the case of *Stichocotyle nephrosis*, in which encysted larvae (or nymphs), with close superficial resemblance to the adults, are present in crabs or lobsters, while the adults occur in the biliary passages of elasmobranchs. It is reasonable to believe that a first-stage larva of this species, as yet undiscovered, develops in some marine mollusc, and later passively or actively reaches the malacostracan host and becomes encysted in the abdomen of this (second?) host. Thus there is cumulative evidence in this group of trematodes, indicating how alternation of hosts is in the process of being achieved, apparently without an alternation of generations. Such a situation more closely resembles that in certain nematodes (spiruroids) than it does a complicated trematode life cycle.

Thus it seems illogical to retain the aspidogastrid species in the subclass Digenea, both because of anatomical and of life-history discrepancies. Likewise they are anatomically disqualified to be returned to the subclass Monogenea. In view of these facts a subclass group intermediate in position between these groups is proposed.

Subclass ASPIDOGASTREA n.n.

Adults parasitic on or in the soft parts of molluscs (gasteropods and lamellibranchs) or in the intestinal canal of cold-blooded vertebrates. Development probably direct, with ciliated or unciliated larva hatching from eggs; adults hermaphroditic. Oral sucker absent or poorly developed; ventral sucking organ a powerful adhesive disc, frequently divided into series of sucking cups; intestine a simple blind sac. Flame-cell pattern of larva: 2 [1 + 1 + 1]. All known species belong to a single suborder, Aspidogastrata Faust, 1932[1], which has the characteristics of the subclass.

[1] Since the genus *Aspidocotyle* Diesing, 1850 is a true digenetic trematode and is unrelated to members of this group, the suborder name Aspidocotylea is unavailable, hence the creation of the name Aspidogastrata Faust, 1932. The subclass name must also be derived from the type genus, *Aspidogaster*.

Furthermore, since the species belonging to the genera *Aspidogaster*, *Cotylaspis*, *Cotylogaster*, *Lophotaspis*, *Platyaspis*, *Macraspis* and *Lobatostoma* all possess more than one row of sucking cups, while *Stichocotyle* has a single row, it is desirable to limit the family Aspidogastridae Poche, 1907, to the former group and to erect a new family, Stichocotylidae, for the genus *Stichocotyle*, which possesses both adequate anatomical and life-history differences to warrant its separation into a distinct family group.

Summary

1. *Cotylaspis sinensis* and *Lophotaspis orientalis* are described as new species of trematodes from the small intestine of the turtle, *Amyda tuberculata*, from Foochow, Fukien Province, China.

2. *Stichocotyle cristata* is described as a new species from the spiral valve of the cow-nosed ray, *Rhinoptera quadriloba*, from Biloxi Bay, Mississippi.

3. A new subgenus, *Multicalyx*, is created for the species *Stichocotyle cristata*, while the species *S. nephropis* appropriately belongs to a new subgenus *Stichocotyle*.

4. Evidence is provided that aspidogastrid worms are referable neither to the Monogenea nor the Digenea, but belong to an intermediate subclass group, Aspidogastrea, n.n.

5. The genus *Stichocotyle* has been removed from the family Aspidogastridae Poche, 1907, and placed in a new family, Stichocotylidae.

Explanation of lettering for figures

AL	acetabular alveoli	*O, OV*	ovary
C	cecum	*P*	papilla of sucking disc
CI	cirrus organ	*PG*	prostate glands
CVD	common vitelline duct	*SR*	seminal receptacle
E	egg	*SV*	seminal vesicle
EB	excretory bladder	*T*	testis
ED	excretory duct	*U*	uterus
GP	genital pore	*V*	vitellaria
MO	marginal organs	*VD*	vitelline duct
OD	oviduct	*VE*	vas efferens
OO	ootype	*VR*	vitelline reservoir

Note. All drawings were made with a Zeiss camera lucida.

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NOTES ON A NEW PROTOZOON, *PIRIDIUM SOCIABILE* N.GEN., N.SP., FROM THE FOOT OF *BUCCINUM UNDATUM*

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(With 15 Figures in the Text)

Introduction

While I was studying the Coccidian *Merocystis kathae*, which lives in the renal organ of the whelk, *Buccinum undatum* (see Patten, 1935), it was often necessary to keep the molluscs in large glass vessels in the laboratory. In October 1933, while the animals were crawling up the sides of the vessel, I noticed white patches 2 or 3 mm. in diameter on the ventral surface of the foot.[1] Microscopic examination showed that each patch was composed of a large number of bodies, undoubtedly a stage in the life history of some protozoon. At first I thought they might be the missing schizogonic phases of *Merocystis*, but later investigations, at other times of the year, showed that was not so. I now believe that the organism which I have found in the foot of the whelk is a schizogregarine. However, the whole life-history does not take place in this site, and I have not yet succeeded in finding the missing stages; nor do those which I have found agree closely with those of any other schizogregarine hitherto described. Until more is known of the life history, the organism can only tentatively be placed among the schizogregarines. Meantime I shall call it *Piridium sociabile*, and shall welcome criticisms and suggestions from other investigators.

Material and methods

The whelks used for this investigation were the same as those used in the study of *Merocystis kathae*. They were generally obtained in Dublin Bay and the routine method followed was to place pieces of foot and renal organ from the same whelk in each jar of fixative. This was done at Coliemore Harbour, Dalkey, to insure that the material should be fresh. As a full description of the methods of fixation and staining were given in my previous paper there is no need to repeat much of the information given there. Bouin's fluid, or Duboscq and Brasil's modification, was the most useful fixative, followed by iron-alum haematoxylin or Giemsa's stain. Sublimate acetic was also used as a fixative, but Feulgen's stain was of little value in studying the parasite of the foot.

Whelks obtained on two occasions from Howth, Co. Dublin, and once

[1] I must thank Mr G. F. Mitchell for first attracting my attention to these structures.

from Plymouth, were also infected with *Piridium sociabile*; but, as far as I know, the incidence of infection is highest in whelks from Dalkey. For instance, notes made on December 14, 1934, of 25 whelks from Plymouth state that only 4 were noticeably infected, while the next day 10 of the 12 from Dalkey were parasitised.

Often whelks were brought to the laboratory and kept in vessels of aerated sea water, so that parasites might be studied alive. To do this, one may either scrape the ventral surface of the foot with a scalpel and examine the material removed in a drop of sea water, or else a piece of the foot may be cut out and teased up on a slide. Similar material was used for making smears. Parasites are found only near the ventral surface of the foot, where they form groups lying immediately under the deep ciliated epithelium.

LIFE HISTORY

In investigating *Merocystis* I found that development followed a seasonal periodicity, and in *Piridium* a similar phenomenon is apparent. Nearly a year is needed for this organism to complete its growth. It will be simplest therefore to begin by describing the smallest stages and to trace their growth and behaviour until they reach that stage when the organism was first discovered, for then it seems to have reached the most mature condition.

In pieces of the foot of the whelk fixed in January and February, groups of minute spherical or oval bodies are seen (Fig. 2) between the cells of the ciliated epithelium. The individual bodies are from 1·8–2·2 μ in size but there is usually little variety in the size of the bodies in a single group. Each contains a conspicuous darkly stained dot, the karyosome of the nucleus, whose outline can only with difficulty be distinguished, though it becomes clearer as growth takes place—as later figures will show. These uninucleate bodies occur singly, or in small numbers, in spaces in a small mass of cytoplasm containing also some large irregular nuclei. These nuclei are very similar in appearance and in staining reaction (*e.g.* the chromatin gives a strong positive reaction with Feulgen's stain) to some of the many types of nuclei that can be seen in the connective-tissue cells of the host. There is little reason to doubt that they are the nuclei of wandering cells which have attacked the group of parasites. What is difficult to determine, is the nature of the protoplasmic network which encloses the parasitic bodies. Is it the cytoplasm of the phagocytes just mentioned or is it an original sheath which surrounded the parasites and in or around which the phagocytic elements have gathered? I think the latter is the more probable explanation; and in that case groups of bodies, and not single bodies, would reach the foot. I have not seen single or even small numbers of these parasites in the epithelial cells, and also the host-nuclei are generally on the surface of the mass as though plastered on to it (as can be seen in Fig. 1).

I think there is no doubt that these groups, lying between the cells of the ciliated epithelium, have entered from the outside and are making their way

to their final position in the subepithelial connective tissue. I have never seen groups actually outside or just piercing the epithelium; nevertheless in many sections made in January, and some in February, all the groups seen are in the epithelial layer—*i.e.* none lie in the connective tissue—and the individual bodies in these groups are always the smallest seen, none being more than 2·2 μ and most 1·8 or 2 μ. Since I have not seen the entrance it is impossible to say whether the group penetrates the epithelium of itself, or whether phagocytic cells pass to the surface and engulf the mass of parasites.

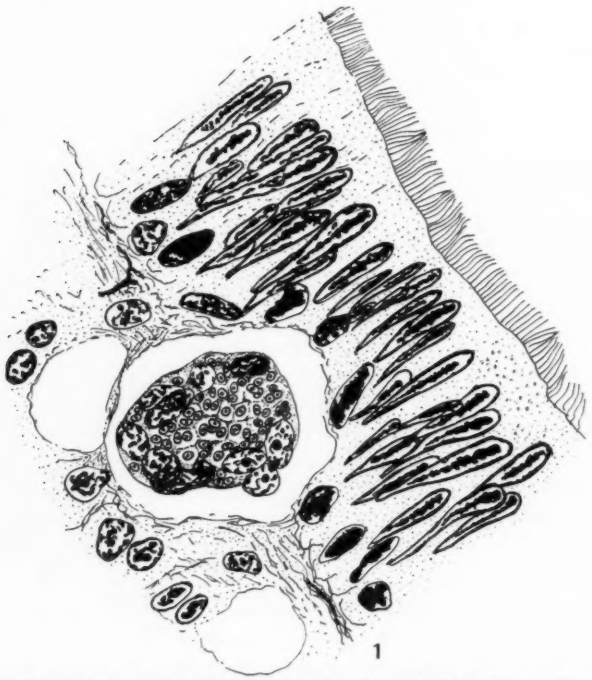


Fig. 1. March. Bouin's fixative, iron-alum haematoxylin stain. × 700.

All drawings were made with the aid of a camera lucida, and a Leitz microscope with Zeiss apochromatic 2 mm. oil-immersion lens. The magnification of all figures, unless otherwise stated, is 1266 diameters. The month in which material was fixed is noted. All preparations, except Fig. 14, were stained with iron-alum haematoxylin.

It seems that connective tissue elements are normally present amongst the cells of the epithelial layer. In other sections made in January and also in the following months conditions such as those shown in Fig. 1 are seen. The clump of bodies now lies partly in the epithelial layer and partly in the connective tissue; other groups can be seen entirely in the latter position (Figs. 3 and 4). By February most of the parasitic masses have reached this, their final position. These figures show that there is little variation in the size of

the bodies in any given group. For example in one slide, the diameter of bodies in 2 groups, still in the epithelial layer, was 1·8 μ; of 3 others in the same position 2·1 μ; while of 4 groups in the connective tissue the size of the bodies in 3 was 3·0 μ, and in the last 3·6 μ.

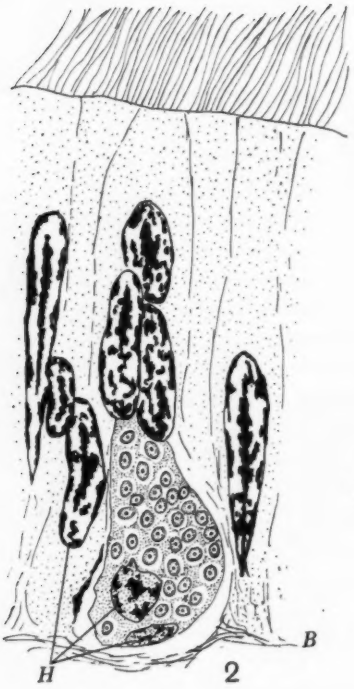
Material was collected towards the end of March in 1934 and 1935, and the condition of the parasites was very similar for both years. (Figs. 4 and 5). In one section examined 18 groups were found, and in all the average size of the bodies is 4·5 μ; but in other whelks earlier stages can be seen, and the parasites are only 2·6 μ. When they are established in their final position in the connective tissue a cyst is formed by the surrounding host tissue.

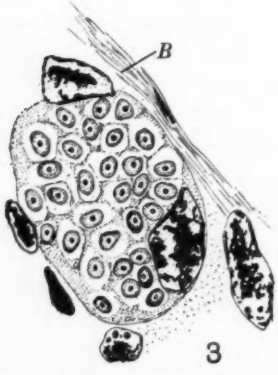
In May the parasites show further growth, as can be seen in Fig. 6, where they measure 8 μ, though in other cysts smaller specimens are found. All cysts are now in the connective tissue. At this date a single deeply staining rod or granule can first be seen close to the periphery in most of the organisms. It is difficult, even in serial sections, to determine whether this granule is present in every body within a cyst, but it probably is. In the figure such a rod can be seen in most of the parasites, and the drawing was made with great care. As later drawings will show, this chromatic rod undergoes much alteration during the growth of the organisms. It is a very characteristic feature of the species, but I am unable to determine its function.

Fig. 6 also shows that the surrounding cyst has grown, and though strands of cytoplasm are seen within, the network which formerly separated the organisms into pockets is breaking down. This process continues and is due to the growth of the organisms.

In June a further increase in the size of the parasites is seen and the chromatic rods are more conspicuous. In the following month whelks were obtained both at Dalkey and at Howth, and there is agreement in the condition of the parasites from the two localities. The most noticeable change is that in many, though not in all cysts, most of the organisms have lost their spherical or ovate shape and are now piriform, their length being 12 or 13 μ and their breadth 7 or 8 μ. The chromatic rod usually lies in the broad end as shown in Fig. 7.

In pieces of foot fixed at the beginning and end of August, the greatest length of the piriform organisms at the earlier date was 15 μ, at the latter 22 μ: but even then the parasites in many cysts were still subspherical. An examination of cysts containing piriform bodies shows that there is a rough orientation of the parasites; generally the broad end, and therefore that part containing the darkly stained rod, lies towards the outside of the cyst (Fig. 8). The rod itself has now a more complex structure, for it is usually club-shaped with the head of the club lying towards the nucleus. The figure however shows two parasites in which this structure looks like a compound body, with the two parts lying almost at right angles to one another. Close to the nucleus, in most of the parasites, a region can be seen which is more finely granular than the rest of the cytoplasm and stains more densely, and when such a region





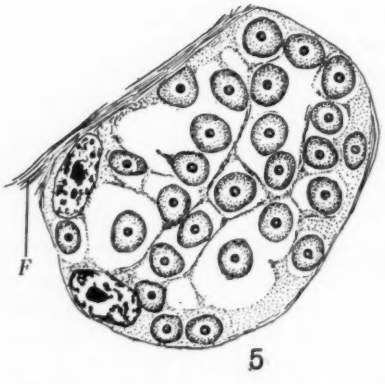


Fig. 2. January. Bouin's fixative. Group of parasites in epithelial layer. H = host nuclei. B = basement membrane of epithelium.

Fig. 3. February. Bouin's fixative. Group in subepithelial connective tissue.

Fig. 4. March. Duboscq and Brasil's modification of Bouin's fixative. Notice small number of parasites outside group and rupture of basement membrane (B). E = nuclei of epithelial cells.

Fig. 5. March. Sublimate-acetic fixation. One of five adjacent groups separated by fibrous membrane (F). Notice breakdown of protoplasmic pockets in group.

is present the deeply staining rod always lies close to it. This disposition becomes more definite later (compare Figs. 12 and 13 with Fig. 8).

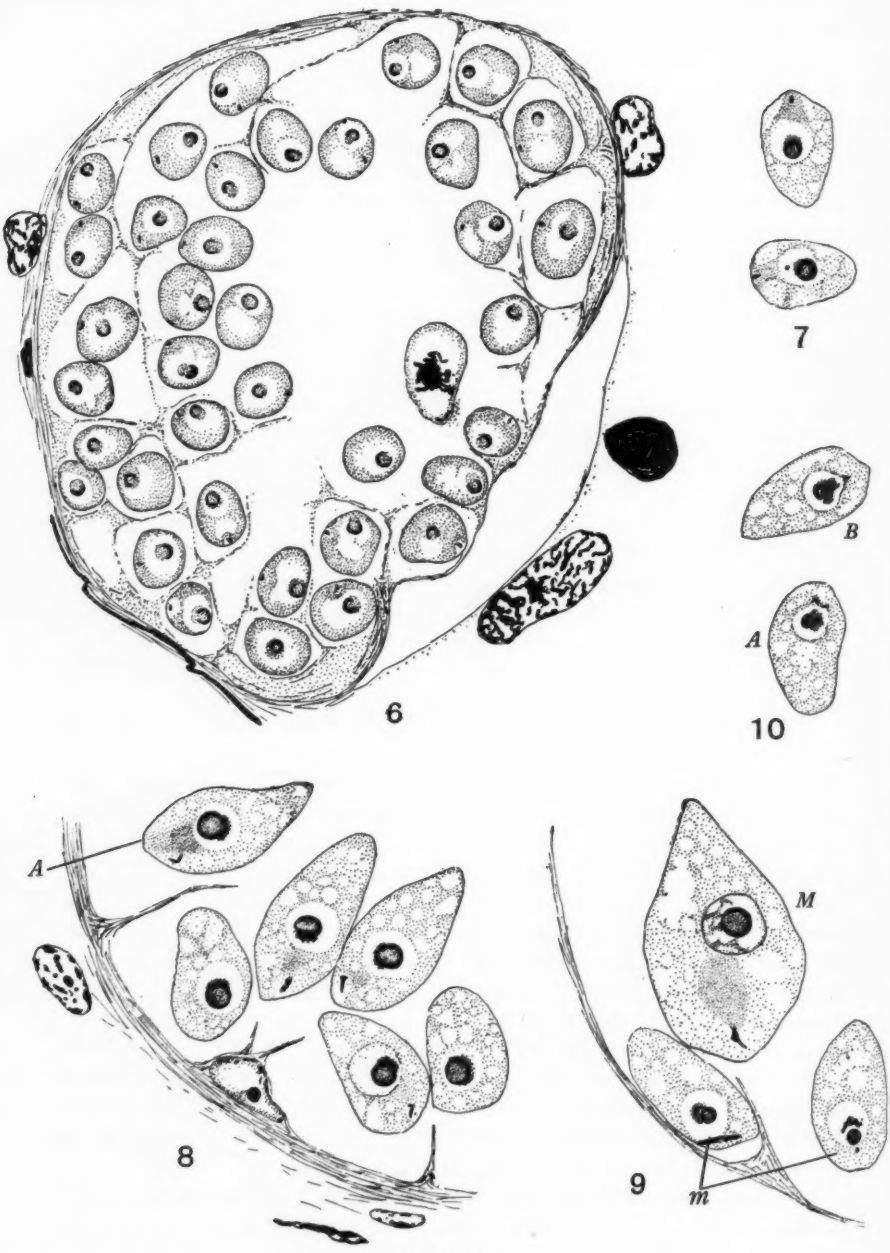
The structure of the nucleus has also changed during the process of growth: the karyosome enlarges and its most densely stained part appears as a peripheral ring; and when it reaches the size shown in Fig. 8 this ring has become thinner and is often incomplete. In earlier stages the rest of the nucleus is homogeneous, but now there is often a mass of deeply stained granules close to the karyosome. This mass gives no mauve reaction with Feulgen's method. In later stages there is sometimes a diffuse pale mauve colour but I have never succeeded in staining a chromatin network. Two of the parasites shown in Fig. 8 have no chromatic rods, but this is not true for the majority of parasites in this cyst, or in other cysts in tissues fixed at the same date. The lack is most likely due to the orientation of the section.

In some cysts fixed at this date there is a true differentiation into two types of body. The larger bodies are like those already described (Fig. 8), but in the same cyst there are others which are somewhat smaller. Their karyosomes are also smaller, and as far as I could decide they are without the dense juxtanuclear area. The chromatic rods are generally present, but neither in position nor orientation so definite as in the larger type.

Later stages in this differentiation may be seen in Fig. 9 drawn from material fixed in September. The maximum size for the larger type has increased up to 30 μ and the shape is now almost that of a pear. The chromatic rod has an appearance suggesting the blepharoplast of Flagellates, and though its structure has not changed greatly, its position appears to be rigidly fixed at the peripheral side of the juxta-nuclear area.

The smaller type of body does not show any increase in size, but a decrease in the size of its karyosome, and no sign of a deeply stained region outside the nucleus. The shape is characteristic, being more fusiform than piriform. In Fig. 10, which is drawn from the same slide as is Fig. 9, peculiar deeply stained strands can be seen attached to the nuclei. In Fig. 10 *A* two such strands are seen while in 10 *B*, which is a profile view, only one could be represented. It seems probable that these strands are derived from the original single chromatic rod, but I have not seen the rod divide. The compound structure seen in two parasites in Fig. 8 does not meet the case, for one of the parasites (*A*) is undoubtedly a young large body. Evidence is rather to be derived from such structures as are seen in Fig. 11. In Fig. 11 *A*, which is a somewhat foreshortened view, the vertical rod probably corresponds to the old rod, while at its distal end is a horizontal bar and from the free end of the latter another vertical rod will probably grow down towards the nucleus. The whole structure would then have such an appearance as is seen in the small bodies of Figs. 12 and 13. Fig. 11 *B* is a lateral view.

It is noticeable that the nuclei of these small bodies, from material fixed in September, are closer to what may be called the anterior ends of the parasites. There is a decrease in the size of the karyosome shown in Figs.



Figs. 6–10.

9 and 10, and a chromatic network is becoming more apparent in the nucleus.

In October, most cysts show this differentiation into large and small bodies, and many large bodies have reached their full size of 40 μ. Incidentally, it was in this month of the year that the parasites were first noticed. Material was collected in 1933 and 1934, both in this month and the next. Fig. 12 shows such a mature cyst but is drawn at a lower magnification than usual in order to show the entire structure, and the orientation and position of the organisms within. It will be seen that the small bodies generally lie near the outside of the mass, and that they have no definite orientation, but tend to lie in groups. The large bodies mostly have the broad end towards the periphery of the cyst. Fig. 13 shows some of the parasites or parts of them at the usual magnification. The chromatic rod in the large bodies has now a peculiar and typical appearance. The inner end is embedded in the juxta-nuclear area, while the outer part curves to make an obtuse angle, and terminates in a fine thread which almost, or quite, reaches the surface. On either side of the angle arise two other, rather thick filaments.

The structure of this complex body might suggest that it really is a blepharoplast and that the filaments are rhizoplasts, but I have examined quantities of these large bodies in stained smears and alive, and I have never seen any free flagella, nor have I seen any movement in the living organisms. When examined alive the structure of the large bodies is seen to be essentially similar to that seen in sections. The blepharoplast-like organ is not visible but in the broad end there is a large dense yellowish-green area. It is larger than the juxta-nuclear area seen in sections and when the parasites are seen end-on it is often ring-shaped. This ring is formed of granules, of varying sizes, of a highly refractile material which appears to be paraglycogen (also abundant in *Merocystis*). The grains can be seen sometimes in fixed material in sections, but particularly in smears. The paraglycogen is best shown, though it is not stained, in preparations stained by Giemsa's method. In Fig. 14 from a smear, it can be seen as a ring round the dense juxta-nuclear area, though scattered granules are also seen in other parts. Small bodies are also shown in this figure and have the usual oval or slightly piriform shape: they have no aggregated paraglycogen granules, and this substance is much less abundant than in the large bodies. Some preliminary experiments with intra-vital staining

Legends to Figs. 6–10

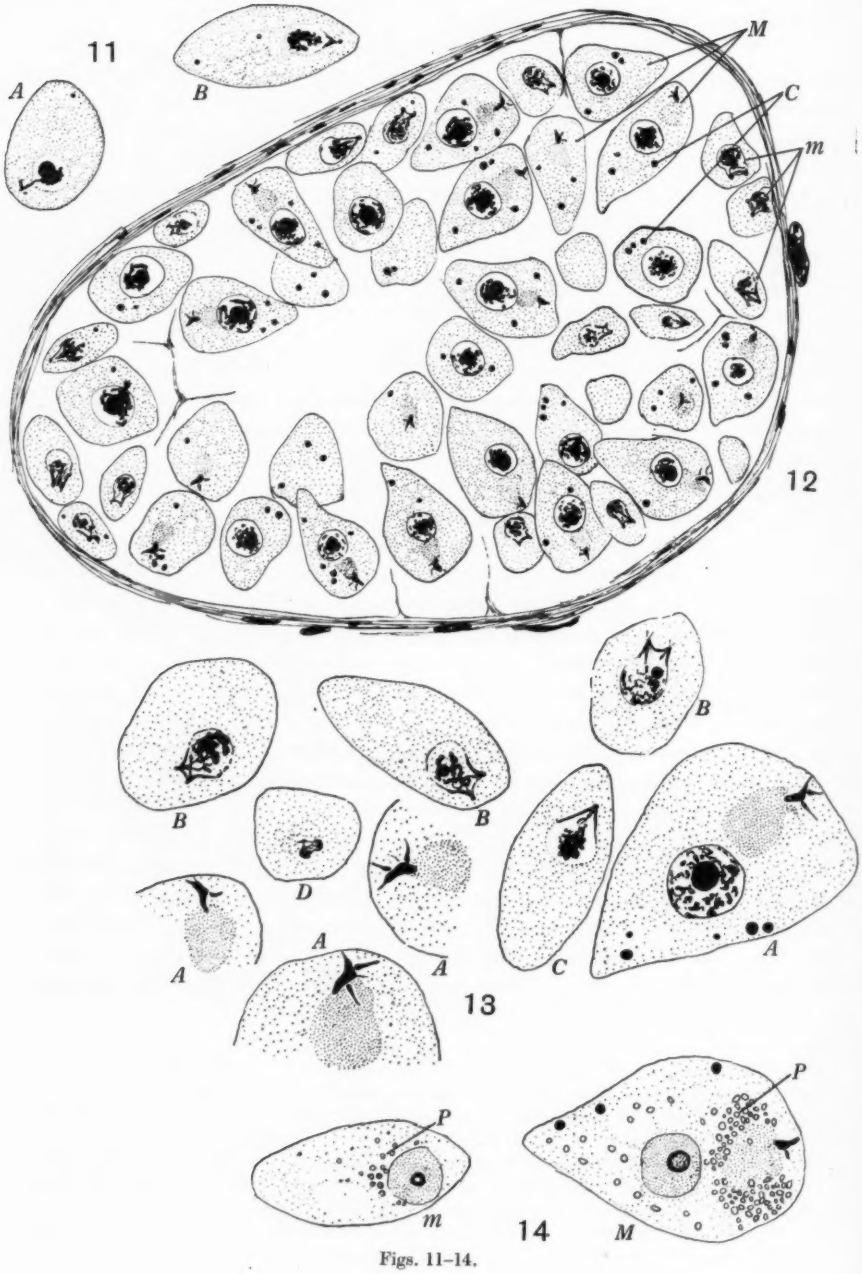
Fig. 6. May. Bouin's fixative. Fibrous cyst formed around group. Chromatic granules in parasites.

Fig. 7. July. Sublimate-acetic fixation. Two piriform organisms from a cyst.

Fig. 8. August. Bouin's fixative. Six organisms and part of cyst wall. Notice orientation, shape of organisms, and chromatic rods in four.

Fig. 9. September. Sublimate-acetic fixation. Differentiation into large and small bodies. Large body (*M*) shows typical position of chromatic rod and dense juxta-nuclear area. In small bodies (*m*) chromatic rod and karyosome small.

Fig. 10. Two small bodies from another cyst in section from which Fig. 9 was drawn. *A*, face view showing pair of chromatic horns attached to nucleus; *B*, single horn in profile.



Figs. 11–14.

with neutral red seemed to indicate that the small bodies can take up this stain readily, while large bodies do not.

Returning to the structure of the small bodies as seen in sections, we find development of the complex structure attached to the nucleus (Figs. 12 and 13). At first I thought that these were forms in which the nucleus was beginning to divide, but this is not the case, or at least if the nucleus does divide it does not do so within the foot of the whelk. I have seen no nuclear divisions whatsoever in *Piridium*.

If the interpretation which I have given for the structures in the small bodies in Figs. 10 and 11 is correct, then the original single darkly stained rod has divided in some way into two, attached by fine cross filaments (Fig. 13 *D*) while the surface of the nucleus comes in contact with this structure and is raised into two horns at either side. From these horns not only do thick rods run down the surface of the nucleus but also fine ones pass into the interior (Fig. 13 *B* and *C*).

I have had much trouble to decide whether all the cysts at some time show this differentiation into the two types of body. In all sections I have found some cysts in which no small bodies could be seen, and this seemed to be true whether growth in size were complete or not. At first I thought that small bodies could not be distinguished till growth was almost complete; but this is incorrect, for a structural differentiation can often be seen earlier, and once an organism develops the typical pear-shape and the juxta-nuclear area, it *is* a large body. Another possibility is that as the small bodies are often in groups, they might be confined to one part of a cyst and therefore missed unless the cyst were followed right through in serial sections, but when this was done there were still many cysts with no small bodies. Moreover, in smear preparations, in which, of course, the organisms are isolated, large bodies are far more abundant than small.

No further development of either type of parasite has been traced in the whelk. It seems probable that the organisms escape from the foot at this time. I have seen many cysts from which the parasites appear to be emerging, and this is not, I think, due to damage done to the piece of foot when removing it. If at the time of year when the cysts are mature one draws a blunt scalpel lightly over the ventral surface of an infected foot there will be many parasites and epithelial cells in the mucus removed. Sometimes even by drawing up

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Legends to Figs. 11–14

Fig. 11. Two small bodies from another cyst in same section as figs. 9 and 10. *A*, foreshortened view; *B*, profile.

Fig. 12. October. Duboscq and Brasil's modification of Bouin's fixative. Section across whole cyst showing large bodies (*M*), small bodies (*m*), and chromatic globules (*C*). ×626.

Fig. 13. October. Sublimate-acetic fixation. (×1626) (i.e. usual magnification). *A*, large body (or part of one) showing structure of chromatic body; *B*, small bodies, face view; *C*, profile view; *D*, end-on view of horns attached to nucleus (which is out of focus).

Fig. 14. October. Bouin's fixative, Giemsa's stain. Smear. (×1626). Large body (*M*) and small body (*m*). *P* = paraglycogen.

water and some mucus in a pipette, from the ventral surface of an undamaged foot, one can see organisms in this material under the microscope. I have not succeeded, however, in finding free organisms in the sea water in which the whelks were kept.

In December both mature cysts and empty or half-empty cysts are common; but in the next 2 months it becomes increasingly rare to find normal cysts, though I once found what appeared to be a healthy cyst containing mature stages as late as March.

Cysts may be attacked by phagocytic cells; such cysts are filled with degenerate and almost unrecognisable organisms and also with masses of host-cells, each containing a nucleus like those described in the early stages. Structures which appear to be cocci and bacilli are often found in these degenerating cysts.

In the hundreds of whelks I have examined I have twice found, in the renal organ, bodies which are very like early stages of *Piridium*. Fig. 15, from a renal organ fixed in May, shows such structures. I presume they are the foot organisms which have reached this site by chance and are degenerating. Both in this and in the other examples mentioned, bacteria-like structures (*B*) are abundant in the kidney close to the parasites. One other structural feature of the large bodies, and to a less extent of the small, must be mentioned: namely, the presence of deeply staining spheres scattered in the cytoplasm of the mature, or almost mature, organisms. They appear to be fairly constant, but stain more strongly after fixation with sublimate acetic or modified Bouin than with the ordinary Bouin method. In Giemsa preparations it can be seen that these "chromatic" granules and the blepharoplast-like rod stain in the same way as the karyosome.

Discussion

It is difficult even to conjecture the affinities of the organism found in the foot of *Buccinum*. I have discovered only that part of the life-history which is passed in the whelk, and though these stages reveal a peculiar cytological structure, they are inadequate to determine the relationship of the organism to other forms.

The most plausible interpretation is that the organism is a schizogregarine,[1] and the groups of minute bodies such as are seen in Figs. 2 and 3 are masses of merozoites which have been set free from some other host. These bundles of merozoites, which are probably surrounded by a protoplasmic membrane, make their way through the ciliated epithelium of the foot of the whelk and in the underlying connective tissue become surrounded by a cyst formed by the host. In this position they undergo growth and development.

In his important work published in 1931, Hatt has described in great detail the method by which the gymnospores of *Nematopsis legeri* and *Porospora gigantea* effect an entrance of the gills, and sometimes the foot, of various

[1] I am greatly indebted to Mr Clifford Dobell, F.R.S., for first suggesting to me that the parasite of the foot might be a schizogregarine.

lamellibranchs and gastropods. These closely related genera can hardly be called schizogregarines for the gymnospores are not groups of merozoites. When the gymnospore, which is set free from a crustacean host, comes in contact with the ciliated epithelium of gill or foot of a suitable mollusc, a wandering phagocytic cell of the host makes its way through the epithelium and engulfs it, and in this condition they pass together to the subepithelial tissue. Each of the minute bodies which compose the gymnospore is then supposed to divide into two. These are the gametes, and they fuse in pairs. In *Porospora* the zygote grows directly into an elongate sporozoite devoid of

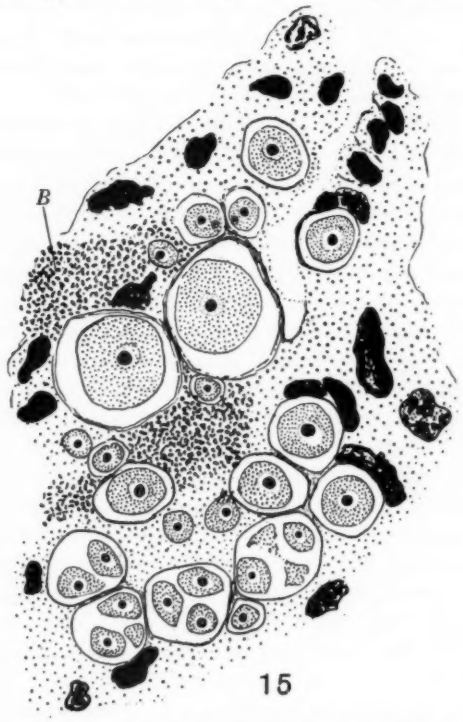

Fig. 15. May. Bouin's fixative, iron-alum haematoxylin stain. Group of bodies resembling young stages of *Piridium* from renal organ of *Buccinum*. *B* = probable bacteria. × 1000.

spore coat, while in *Nematopsis* a spore is formed containing a single sporozoite. M. Hatt has been able to follow the whole history in both these genera by means of infection experiments. The cycle takes a comparatively short time.

There are some points which suggest a relationship between *Piridium* and the *Porospora* group. First, both pass part of the life-history in molluscan hosts, and Hatt found that, though the gill was the part most frequently infected, *Porospora* is also capable of developing in the foot. And secondly, an examination of his figures of *P. gigantea* shows certain morphological re-

semblances to *Piridium*. The zygote and young sporozoites are somewhat like the bodies seen in my Figs. 2 and 3: while, Fig. 8 (Hatt), in the tip of a young sporozoite is a darkly stained granule which suggests the rod in *Piridium*.

It seems to me that there is no close homology, however. The minute bodies which enter the foot of *Buccinum* do not divide, nor do they fuse (before further growth) to form zygotes. In order to homologise *Piridium* with *Porospora* it would be necessary to postulate that the minute bodies, in the group which enters the whelk, are zygotes; but in that case one would expect such zygotes to grow into sporozoites, whereas they develop into organisms up to 40μ in size having no resemblance to sporozoites.

I have examined the gills and various other parts of whelks on many occasions, and except for the two cases mentioned above (in the renal organ), have found nothing resembling the parasites of the foot.[1]

I have seen most of the literature on the schizogregarines (for the most recent reviews see Wenyon, 1926, and Naville, 1931). The species recently investigated seem to be sufficiently fully described to show that they have no close affinities with *Piridium*. More hopeful are the peculiar and little known organisms found in Annelid worms—*Joyeuxella toxoides* (Brasil, 1902), *Eleutheroschizon duboscqi* (Brasil, 1906), *Schaudinella henleae* (Nusbaum, 1903; see also Minchin, 1912), *Siedleckia nematoides* (Caullery and Mesnil, 1899), and *Spirocystis nidula* (Léger and Duboscq, 1915). In the first four schizogony and little else has been described, but *Spirocystis* seems to me to have affinities with *Piridium*. In *S. nidula* the "schizont" becomes spirally coiled, and the "merozoites" which it produces are supposed to grow into broods of gametes. The macrogametes are ovoid or globular, while the microgametes are narrower and fusiform and their cytoplasm is alveolar, whereas in the former it is densely coloured and has many grains, probably mitochondria. Generally there is also a long thread coiled about the nucleus, and looking as though it might have some connection with the nuclear spindle, but the authors consider it to be of mitochondrial origin. The number of gametes in a brood varies, but is usually from 5 to 12. Fusion of these bodies was not seen, but isolated spores, very like those of *Nematopsis*, containing one twisted sporozoite, were common.

It seems to me that there are many features in common between *Spirocystis* and *Piridium*, and I tentatively suggest that the larger and smaller bodies of the parasite of the whelk are also to be looked upon as sexually differentiated structures, either gametes or gametocytes. Even in shape they have resemblances to the gametes of *Spirocystis*. In both species the smaller body is ovoid or fusiform and proportionately narrower than the larger; the latter in *Spirocystis* is globular, while in *Piridium* it is pear-shaped. The thread-like structure so typical of the macrogamete of *Spirocystis* suggests comparison

[1] *Cardium edule* from Dublin Bay is infected with *Nematopsis—N. portunidarum*, I presume. I have examined a few littoral gastropods from Dalkey, but have found no infection with *Nematopsis* or *Porospora*.

with the blepharoplast-like structure with its filaments in the parasite of the whelk. It is of interest to note that Léger and Duboscq say "Sans doute, et sans être très étroites, les affinités les plus proches du *Spirocystis* semblent être avec les *Porospora*."

One difficulty in the hypothesis that the size differentiation seen in the mature forms of *Piridium* is sexual, lies in the fact that the large bodies are so much more abundant than the small ones, which, as I have already shown, appear to be lacking in many cysts. It is improbable that fusion between the "gametes" has already taken place in such cysts, for the appearance of the organisms within is identical with that of ordinary large bodies. At present I can offer no explanation for this anomaly.

It seems probable that both types of organism escape into the sea where they have a free-living existence for a time and may produce sporozoites. In any case the organism probably reaches a different host, in which schizogony occurs and bundles of merozoites are produced which in turn infect the whelk. As far as I know, no organism has been described which seems to supply the missing schizogonic cycle of *Piridium*. The organism should be common, judging by the frequency and abundance of infection of *Buccinum*.

In conclusion I should like to mention the interesting work done by Ray (1930 *a*) and Mackinnon and Ray (1933) on various species of "*Selenidium*". In these papers it was shown that peculiar chromatic thread-like structures are seen in the anterior end of *Selenidia*, and that their number, type, and length are fairly constant for any given species. Ray remarks on the probable importance of these morphological features in determining the relationships of the group, and notes that the threads give the same staining reactions as the karyosome. Their appearance suggests the deeply stained rods of *Piridium*; and although this organism cannot be closely related to "*Selenidium*", it may be useful to stress these outstanding morphological features, and to suggest that they may have counterparts in other gregarines. The scattered chromatic globules seen particularly in mature large bodies remind one of those figured by Ray in the coccidian *Dorisiella scolelepidis* (Ray, 1930 *b*).

I hope to continue the investigation of *Piridium*, and if I can find out how to keep whelks alive and healthy in the laboratory it ought to be possible to fill up some of the gaps in the life-history.

SUMMARY AND DEFINITION

Piridium n.gen.

The earliest stages found are groups of small bodies, 1·8 or 2·0 μ in diameter. These groups are probably taken up by phagocytic cells and pass through the epithelium of the host into the connective tissue, where a cyst is formed by the host. Within the cyst the bodies grow. In many cysts there is a differentiation into two types: the larger, when mature, attain 40 μ in size, are shaped like a pear, and contain at the broad end a chromatic rod. The small bodies

reach 30μ in length, are also somewhat piriform, and contain two similar structures attached to their nuclei. In some cysts large bodies alone are found.

Development is periodic, the youngest stages being found from January to March and the parasites not reaching maturity until September–November.

Type species *P. sociabile*, with the features mentioned above: found near the ventral surface of the foot of *Buccinum undatum*.

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ON A NEW TYPE OF RESPIRATORY INTERRELATION BETWEEN AN INSECT (CHALCID) PARASITE AND ITS HOST (COCCIDAE)

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(With 24 Figures in the Text)

CONTENTS

		PAGE
I.	Introduction	517
II.	Life history of *Encyrtus infelix*	518
	(1) The egg	518
	(2) First-instar larva	520
	(3) Second-instar larva	520
	(4) Third-instar larva	520
	(5) Fourth- and fifth-instar larvae	522
	(6) The sheath enclosing the larva	522
	(7) The pupa and its sheath	523
	(8) Mode of development of the sheath	525
	(9) Adult	527
III.	Discussion of the interrelation between parasite and host	527
	(1) Significance of the sheath	529
	(2) Histology of healthy *Saissetia* in relation to sheath formation	531
	(3) Significance of the tracheal attachments	532
IV.	"Puparia" and sheath formation in other chalcid parasites	536
V.	Summary	538
	References	539

I. Introduction

In 1902 Miss A. Embleton described a new species of chalcid parasite belonging to the genus *Comys*. The material was obtained from specimens of the scale insect *Saissetia* (*Lecanium*) *hemisphaerica* (Targioni) which were infesting a variety of ferns (*Asplenium*) and other plants in the Cambridge Botanic Gardens. It can also be found attacking *Coccus hesperidum* L., but this is an unusual host. In 1904 Embleton published a brief account of the biology of the insect, together with a description of some of the early stages and a detailed account of the structure of the adult; which account, although incomplete and obviously incorrect in many respects, drew attention to some remarkable peculiarities of the respiratory system of the larva and its relation with that of the host. She described a membrane enclosing the pupa and what appeared to be an external tracheal system projecting outwards from the spiracles of the fourth-stage larva and ramifying among the organs of the

scale insect. It was clear from this paper that the biology of this species was of quite extraordinary interest, and accordingly efforts were made to secure an adequate supply of material so that the whole life-history could be worked out in detail. Material was obtained from the Botanic Gardens at Cambridge, and also from the Royal Botanic Gardens at Kew. A culture of the host *S. hemisphaerica* was then established at the Cambridge Entomological Field Station on *Ardisia crispa*, and the adult parasites were introduced. The insects bred readily and a very high percentage of parasitism was quickly obtained; and in this way material of all the different stages in the life history was available whenever required.

The host insect, *S. hemisphaerica*, attacks a very great variety of plants and is to be found in many greenhouses and hothouses in this country. It appears to be distributed throughout the subtropical regions of the world and has also been recorded from the Tropics proper (Ceylon, Brazil and the East Indies), but its place of origin is uncertain.

Comys infelix does not appear to have been recorded outside the British Isles, although it can hardly be a native insect. The genus *Comys* was established by Förster in 1856 to replace the genus *Eucomys*, a name which was thought to be already preoccupied. *Comys* has since been synonymised with *Encyrtus*, and accordingly the subject of this paper should now be known as *Encyrtus infelix* (Embleton). Species belonging to the original genus *Eucomys* have been described from Europe, Africa, the Canary Islands, North and Central America, and Australia. They are known to parasitise scale insects of the genera *Saissetia*, *Eulecanium*, *Pulvinaria* and *Ceroplastes*.

II. Life history of Encyrtus infelix

(1) *The egg*

The ovarian egg of *E. infelix* has already been described by Embleton (1904) and that of *E. infidus* by Silvestri (1919). It is roughly of a dumbbell shape, two masses of yolk being connected by a narrow isthmus. As development proceeds one of the yolk masses is rapidly absorbed by the future egg proper, and at the time of oviposition the egg is oval in shape with a short stalk having a cup-like structure at the anterior end. This is often partially plugged by the remains of the apical yolk mass. The body of the egg is between 0·4 and 0·5 mm. long and the stalk about 0·25 mm. The egg is inserted by the female under the dorsal body wall of a second-stage host, the stalk being left protruding to the exterior (Fig. 1). Although the eggs may be sometimes found at the anterior end of the host, more than 90 per cent. are deposited in the posterior region, usually just to one side of the anal cleft. Development proceeds rapidly, blastoderm formation being completed in about 24 hours at a constant temperature of 25° C. and the larva is ready to hatch on the fourth or fifth day. Although a large number of eggs may be laid and will hatch within a single host, I have never known more than one larva reach maturity;

in the vast majority of cases all the larvae save one succumb in the course of the first instar.

(2) *First-instar larva*

The first-instar larva is illustrated in Figs. 2 and 3, and does not require a detailed description. The respiratory system is metapneustic and the tenth postcephalic segment of the body is prolonged in the form of two processes, each bearing an open spiracle at the tip. The length, exclusive of these processes, is approximately 0·6–0·75 mm. soon after hatching. The larva remains attached to the empty chorion of the egg, the caudal processes apparently being firmly fastened at a point on the internal surface of the egg wall just below the stalk (Fig. 4). A conspicuous finely shagreened band reaches from the stalk almost to the extremity of the egg, and the chorion in this region appears to be of a spongy nature. The stalk itself is hollow, and by this method the larva is enabled to utilise atmospheric air. A similar condition has already been described in allied genera and is probably not infrequent among chalcid parasites of scale insects (see Imms, 1918). That the spiracles are actually open and in communication with the air is shown by the following experiment, employing a simple technique based on the observations of Freeborn & Atsatt (1918) on mosquito larvae.

If a scale insect containing a first-stage parasite larva is placed in a thin vegetable oil which has been strongly stained with a dye such as Sudan III, it will be found that the oil speedily enters the egg stalk and, owing to the hydrofuge nature of spiracle structures and tracheal walls, invades the tracheal system of the larva. Dissection then reveals the tracheal trunks of the parasite completely injected with the coloured oil.

(3) *Second-instar larva*

The distinguishing characters of the second-instar larva are shown in Fig. 3 B. The larva needs little detailed description, for it is of the same general form as the last. The length of the larval body varies between about 0·9 and 1·2 mm. The caudal processes are somewhat longer, allowing more scope to the larva which is feeding on the blood and fat body of the host, but they are still firmly attached as before. The first larval skin is not completely cast: it is merely shuffled off backwards and remains loosely attached around the base of the "tails".

(4) *Third-instar larva*

The third-instar larva is illustrated in Figs. 5 and 3 C. Its mode of life is essentially similar to that already described, save that the caudal processes are very much elongated. Fig. 5 shows a specimen which has been pulled away from the egg in order to show the full length of these appendages and the position of the cast skins of the first two instars, the head capsules of which can be clearly seen. The later stages vary greatly in size according to the size of the host which they inhabit. The third instar varies from 1·2 to 1·9 mm. in length of body.

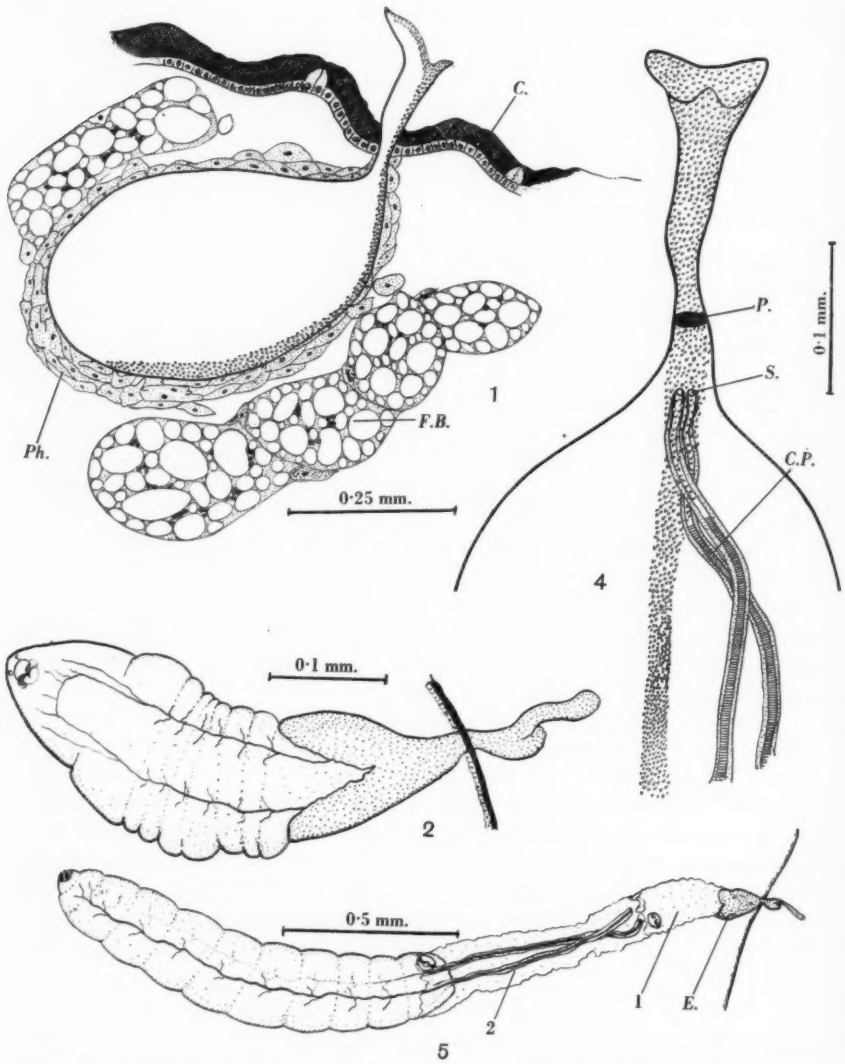


Fig. 1. Semi-diagrammatic section of egg in position with stalk projecting through the dorsal body wall of the host. The egg from which the drawing was made was moribund and was consequently surrounded by a dense envelope of phagocytes.

Fig. 2. First-instar larva attached to empty chorion of egg.

Fig. 4. View of upper part of chorion of egg showing the shagreened stalk; the shagreened strip running down the egg and the attachment of the caudal processes of the larva to the chorion.

Fig. 5. Third-instar larva which has been partially pulled away from the egg in order to show the length of the caudal processes and the position of the cast skins of the first two instars.

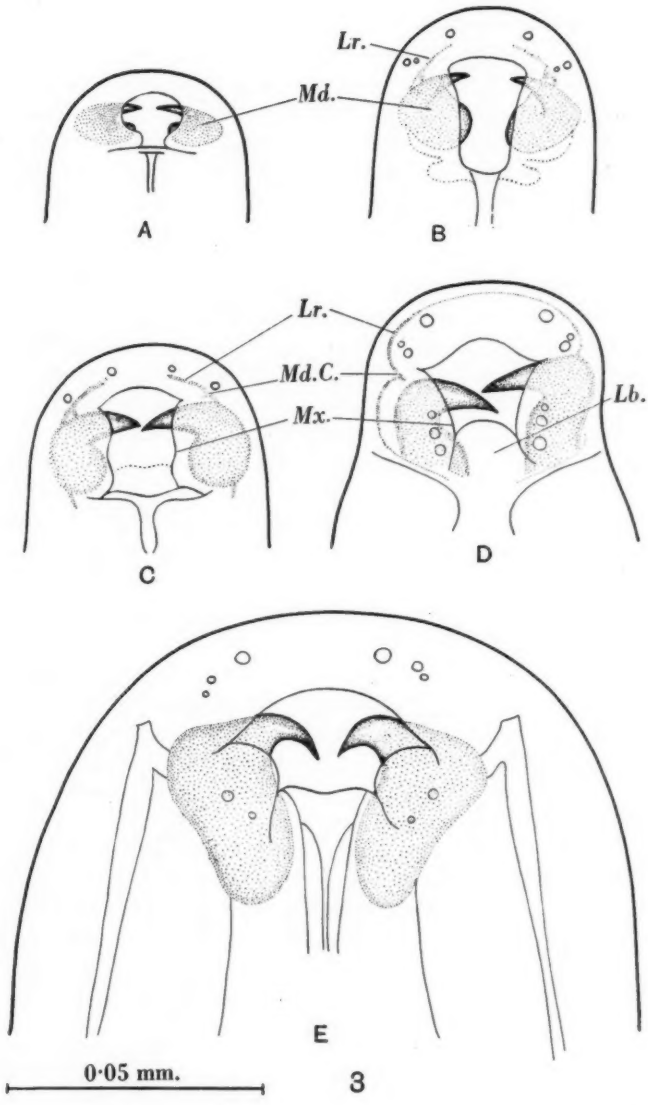


Fig. 3. Mouth parts of larval stages in ventral view. Drawn from cast skins. A, first instar B, second instar. C, third instar. D, fourth instar. E, fifth instar.

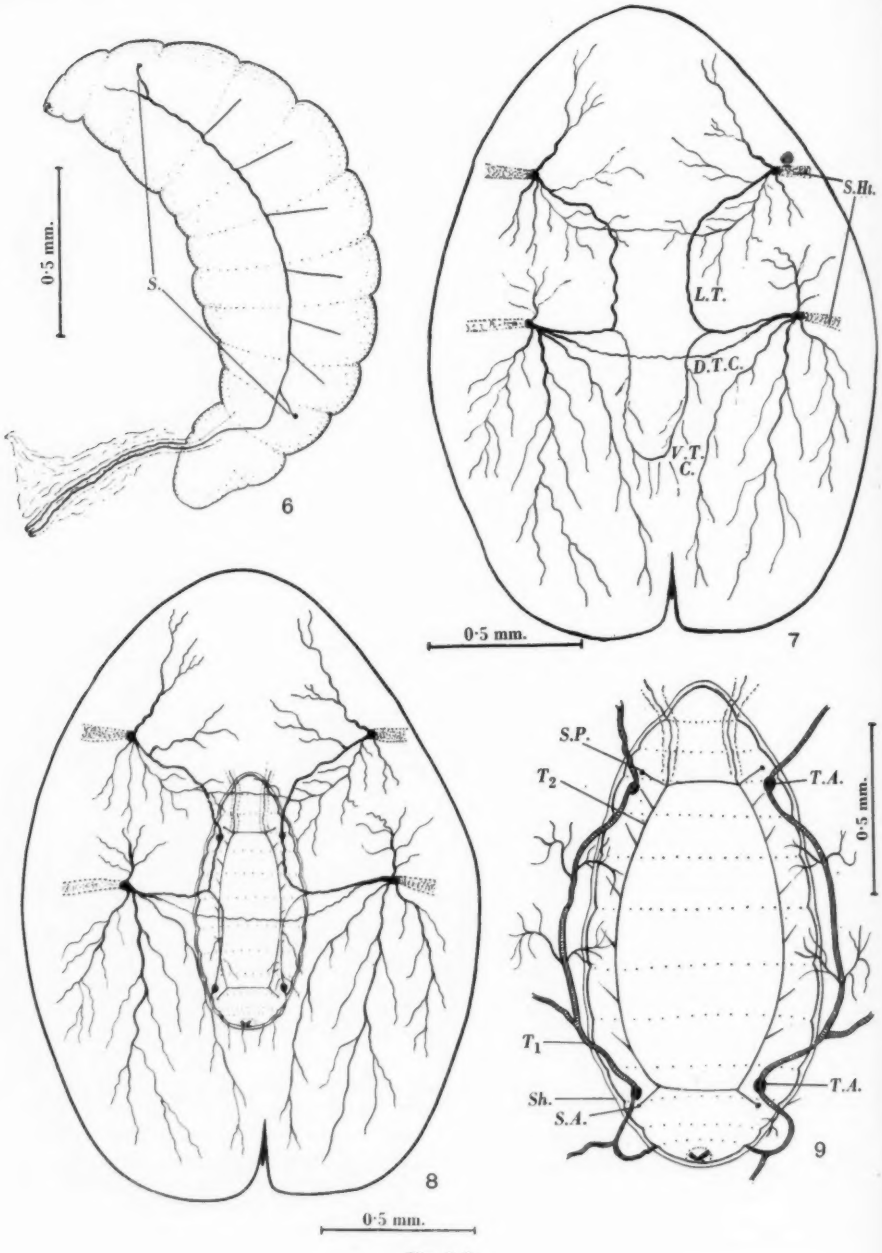


Fig. 6–9.

(5) *Fourth- and fifth-instar larvae*

At the beginning of the fourth larval stage the attachment and mode of life is as before, but in addition to the open spiracles inserted into the egg stalk two extra pairs of spiracles are present. One of these pairs is situated on the *first* thoracic segment and the other on the penultimate segment (Fig. 6). In addition there are now six conspicuous branches arising from the lateral tracheal trunks. These run to the pleural regions of their respective segments, but lack spiracles. The body of this stage may vary between 1·6 and 2·3 mm. in length. The head capsule is shown in Fig. 3 D.

The tracheal system of unparasitised scale insects at this stage is illustrated in Fig. 7, and at this period a remarkable change in the habits of the parasite takes place. Although still attached to the egg by the "tails" the larva now turns round within the host[1] and arranges itself with the head pointing towards the posterior region of the scale and the anterior segments resting on the posterior tracheal commissure, the sides of its body being closely apposed to the main longitudinal trunks of the host. It now lies alongside and slightly above the great rectal chamber of the scale insect and beneath the "supra visceral" membrane (Fig. 20). Shortly after this change in position another moult takes place and the larva enters upon its final instar (Fig. 3 E). This larva is similar to the last save that the caudal processes are degenerate and sooner or later the connection with the egg-stalk is lost.

(6) *The sheath enclosing the larva*

If the larva is dissected out at this time, a very remarkable state of affairs is observed. The parasite appears to have grown an external tracheal system which branches out from four points on its body wall near to, but not coincident with, the four open spiracles. Actually what has happened is that the larva has become invested by a very delicate, tightly fitting, transparent sheath, to which the main tracheal trunks of the host have become firmly attached in four or six places. The points of attachment, as will be seen from the illustration, are marked by the development of strongly sclerotised and pigmented knobs. These may appear to be fused to the skin of the larva, but this is not the case, for with care the "knobs" together with the sheath can

[1] That this turning movement is the result of an innate instinct and is not dependent on some stimulus provided by the tissues of the host is shown by the fact that even in those rare cases where the egg has been deposited anteriorly the tendency to turn is still manifest.

Text to figs. 6–9.

Fig. 6. Fourth-instar larva. The "caudal processes" now show signs of degeneration. The tracheal system is amphipneustic. The finer tracheal branches are omitted in the figure.

Fig. 7. Young scale insect (unparasitised) dissected from dorsal side to show the main features of the tracheal system.

Fig. 8. Ditto with fifth-instar parasite larva *in situ*.

Fig. 9. Fifth-instar larva dissected out still enclosed in its sheath together with the longitudinal tracheal trunks of the host attached.

be detached, leaving the larval cuticle intact beneath. The attachment of the tracheae to the sheath is, however, so strong that in dissection the tracheal trunks themselves break rather than become detached therefrom. Consequently if the dissection is not done with the utmost care the true nature of the tracheae is not apparent and they appear to constitute an external tracheal system belonging to the larva itself, the sheath at this stage being extremely difficult to see. The sheath, which is at first incomplete, soon spreads to form an entire envelope and rapidly becomes tough, showing pigmentation dorsally. By this time it is quite easy to dissect it off, and this has been done in a large number of cases in order to study the chemical composition of the membrane of which it is composed. At this stage the membrane appears quite structureless both when mounted whole and when seen in section. It was supposed at first that the sheath consisted, at least in part, of an additional larval skin which instead of being cast in the normal way remained intact as a sort of puparium. This was soon found to be incorrect. Scores of sheaths were dissected off and mounted, but in no case could any trace of the larval mandibles, head capsule or spiracles be found to form part of them; although cast skins with their mouth parts may often be found loosely attached to the sheath externally. Secondly, while it is very easy to demonstrate the presence of chitin in a cast larval skin, no chitin reaction can be obtained with the sheath. The sheath is waxy in nature and appears to contain a great deal of "cuticulin-like" substance. The presence of protein is also clearly demonstrated by means of the Biuret test: the thin ventral part of the sheath appears to dissolve entirely when subjected to very hot caustic potash and probably consists of protein only. Thirdly, the parasite completes its final moult within the sheath, and the larval skin and head capsule belonging to this instar can always be found enclosed within it at the posterior end of the pupa. Fig. 10 shows a parasite in early pupal stage contained in its sheath and viewed from the side. The two lateral spiracles of the host and the point of tracheal attachments are indicated diagrammatically.

(7) *The pupa and its sheath*

The fifth-instar larva before long begins to show indications of the developing imaginal organs (prepupal stage), which become visible through its transparent skin and the investing sheath. Finally the last larval skin is cast and can always be found enclosed within the sheath at the hind-end of the pupa (Fig. 12). The pupa may vary in length from 1·8 to 2·3 mm. according to the size of the host. Shortly before pupation bubbles of gas are seen within the sheath just underneath the points of tracheal attachment. These bubbles increase in size at the time of pupation and extend in a somewhat irregular manner along the folds and grooves of the pupal body. The pupa itself has three pairs of spiracles as shown in Fig. 11, the anterior and posterior pair being clearly open, the middle pair in all probability closed. Each posterior spiracle lies at the end of a shallow groove or vestibule (Fig. 13) beset with

fine spines which entangle the air bubble and have the appearance of being specially adapted for the purpose, the bubbles thus extending right to the spiracle itself. Similarly the anterior bubbles are also in actual contact with the anterior spiracles of the pupa, and these facts suggest that actual perforation of the host trachea has taken place at the points of attachment, and that although there is no *structural* continuity between the respiratory system of the two insects, the parasite spiracles have thus been put into *functional* communication with the host tracheal system. The whole structure has in fact the appearance of being an elaborate method of supplying the fifth larval and the pupal stage with air after the connection with the egg stalk has been broken.

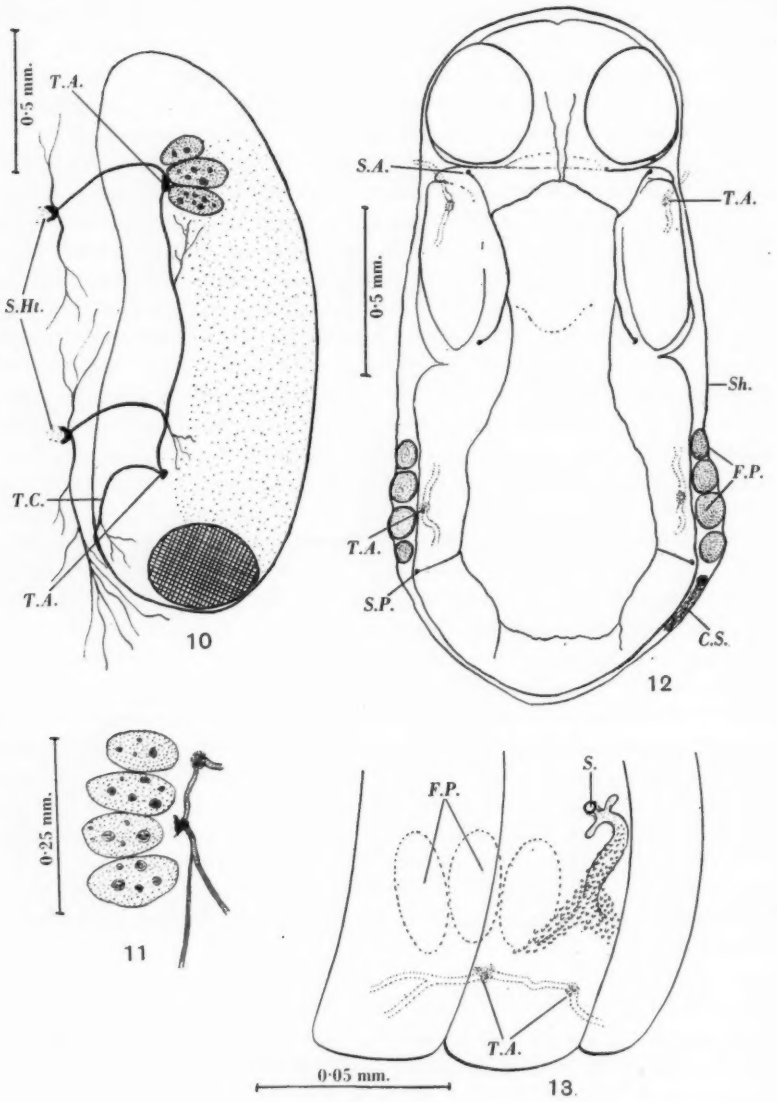
That there actually is functional continuity has again been proved by the oil Sudan III method. If an intact scale insect containing a parasite in the early pupal stage is placed in oil, the fluid will rapidly penetrate the tracheal system of the host by way of the spiracles and if after 2 or 3 hours the contained parasite is then dissected it is found that the oil is not only deeply injected in the tracheal system of the host but has penetrated the tracheal attachments into the sheath, has displaced the bubbles of air therein and has entered and completely injected the tracheal system of the pupal parasite.

It is evident that, in correlation with the provision of a direct air supply via the host tracheal system the typical peripneustic tracheal system of a full-grown chalcid larva with its nine pairs of spiracles never develops. All save two pairs of spiracles are lacking although the lateral tracheal branches that supplied them remain.

Just above the posterior tracheal attachments three curious oval brown bodies are to be seen (Fig. 11). They lie within the sheath, lightly attached to its wall and are almost always symmetrically placed. They contain a very large number of granules of different sizes, the larger ones being concretions of uric acid. They give a positive reaction to the test of Benedict and Hitchcock (1915). They are in fact nothing more than the faecal pellets extruded by the larva just prior to pupation. The constancy of position is remarkable, and still more curious is the fact that they become attached to the internal wall of the sheath, so that when this is dissected off they come away with it, thus giving the impression of being part of the sheath itself. Their position is also indicated in Figs. 10, 12, 16 and 17.

(8) *Mode of development of the sheath*

Serial sections through scale insects containing parasites in the first, second, or early third stage show no striking reaction on the part of the host tissues. There is no marked proliferation of the hypodermis at the point of insertion of the egg, and phagocytes are not particularly in evidence, although a few may be seen. If, however, an egg has failed to develop or a first-stage larva has died, then a conspicuous phagocytic envelope three or four cells thick may often be observed (see Fig. 1). On the other hand, if a scale insect containing a late third- or early fourth-instar larva is sectioned, one finds traces


0·5 mm.
T.A.
S.Ht.
T.C.
T.A.
10
S.A.
T.A.
0·5 mm.
Sh.
F.P.
T.A.
S.P.
C.S.
12
0·25 mm.
11
F.P.
S.
T.A.
0·05 mm.
13

Figs. 10–13.

of an extremely thin membrane partially surrounding the parasite. At this stage it is much too delicate to be distinguished by dissection. It is not a complete envelope and in many places is seen to be confluent with the normal connective tissue membranes surrounding the fat body, alimentary canal and other organs of the host (see Fig. 14). The work of Lazarenko has recently thrown a great deal of light on the difficult question of the origin of the connective tissue membranes and basement membranes in insects. He has shown that these membranes are produced by phagocytes which have a tendency to flatten themselves out on smooth surfaces in the form of a web. These phagocytes quickly lose their nuclei, with the result that a very thin, and apparently structureless, membrane is formed. Similar observations have been recorded by Wigglesworth in the course of his studies on the development of *Rhodnius*. This is evidently the first step in the development of the sheath surrounding the larva of *Encyrtus*. In the early stages of sheath formation there are no signs of any tracheal attachments, although fine tracheal branches may be seen running over the membrane, here and there appearing to coalesce with it.

These fine tracheae proliferate and thus play an important part in the extension and completion of the membrane. Their activity in this respect recalls the sheath production by the tracheae of insects parasitised by tachinids, but in the latter case it is a single large trunk, which by *mechanical* stimulus is made to "pour out" a membrane-forming secretion over the parasite (Pantel). In the case under discussion it is the fine tracheae which are apparently subject to *physiological* stimulation causing them, together with the phagocytes, to form a similar transparent and seemingly structureless membrane.

After the sheath has been completed anteriorly the head of the larva can be observed twisting and turning inside it, the mandibles in constant motion as if in the attempt to break through. But the membrane continues toughening and thickening somewhat, particularly in the dorsal region which, by the time the parasite has reached the pupal stage, has become deep brown in colour.

From the first the activity of these tracheae and phagocytes is most marked in the region of the parasite spiracles and the membrane here almost immediately becomes thickened to form a brownish patch. At the same time the two main lateral tracheal trunks of the host become sharply bent inwards so

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Text to figs. 10–13.

Fig. 10. Diagrammatic representation of an early pupa dissected from the host but with sheath and tracheal attachments still intact. The left spiracles of the host are indicated. The sheath now shows faint pigmentation dorsally; faecal pellets have been extruded and the developing compound eye of the adult is visible.

Fig. 11. Group of faecal pellets showing concretions of uric acid. A double tracheal attachment is also shown.

Fig. 12. Diagrammatic optical section of pupa in its sheath, to show faecal pellets, cast skin of fifth instar, position of tracheal attachments and pupal spiracles.

Fig. 13. Abdominal spiracle of pupa, with its grooved vestibule, viewed through the sheath. The position of faecal pellets and tracheal attachments is shown by broken lines.

as to be in contact with the thickened patch. This patch is the brown knob-like structure which is so conspicuous on dissection and which, as will be seen from Fig. 16, projects inwards against the body of the parasite. Although many sections show an actual break in the tracheal trunk at this point, it would be difficult to be positive that a connection had been established, were it not for the experiments with the oil above mentioned. In addition to this breaking of the main tracheal trunk, several small branches may arise at the point of attachment and run into the sheath through the substance of the knob. The section illustrated in Fig. 16 shows well the activity of phagocytes and tracheal epithelium at this point.

(9) *Adult*

The pupal stage lasts about a week at an average temperature of 65–70° F. and the adult, when ready to emerge, makes a neat circular aperture with its mandibles through both the sheath and the dorsal body wall of the host, which is thus killed before it has been able to produce any eggs. The duration of life of the adult *Encyrtus*, which feeds on "honey dew" secreted by the scale insect, varies from about 10–14 days at a temperature of about 65–70° F. The structure and habits of the adult chalcid have already been well described by Embleton.

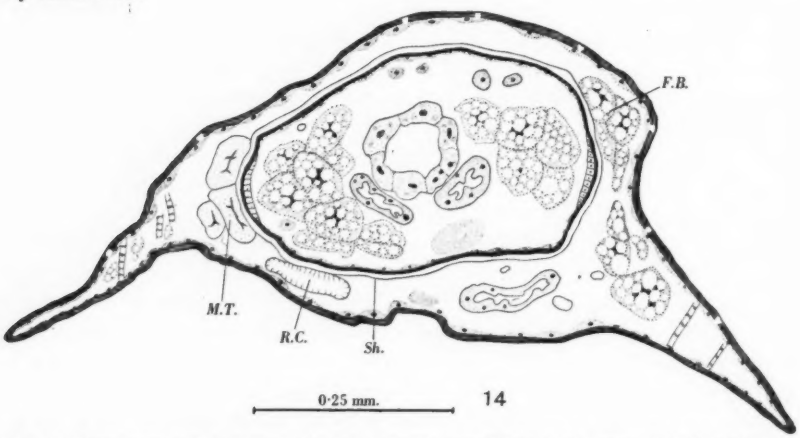


Fig. 14. Transverse section through young *Saissetia* containing third instar *E. infelix* showing early development of sheath and its attachment to various organs of the host.

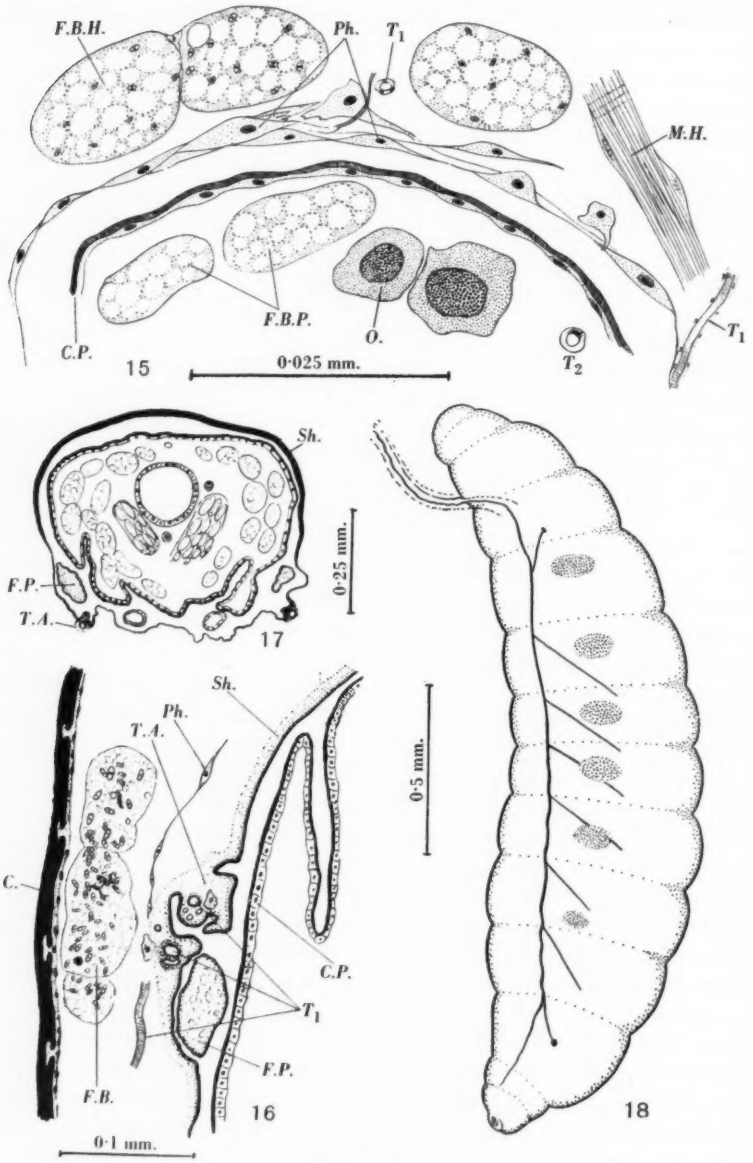
III. Discussion of the interrelation between parasite and host

It will be obvious that a study of the life history of such an insect raises problems of great theoretical interest. Here we have a parasite which suddenly, at a critical stage in its life history, changes its manner of respiration and becomes dependent on an elaborate structure developed by its host—a structure which, it seems, can be of no value whatever to the animal producing it, but which has every appearance of being developed especially for the well-

being of the parasite. It is as if the parasite soma suddenly became endowed with the power of organising and dominating the activity of certain tissues of its host, causing them to develop solely for its own convenience. Such a state of affairs, though without parallel among parasitic insects, recalls the extraordinary Copepod, *Xencoeloma* (Caullery & Mesnil, 1919), parasitic in *Polycirrus*, in which there is a real mingling of host and parasite tissue, both structurally and functionally. But one must first consider whether any simpler explanation can be put forward. For the purposes of discussion it is convenient to consider sheath formation and tracheal attachment separately.

(1) *Significance of the sheath*

Obviously the formation of an inflammatory sheath in response to parasitic infection is not in itself a very surprising phenomenon, and it does not seem necessary to invoke any special explanation to account for this particular case. The production of such a sheath by the host is quite intelligible in so far that any process which tends to separate a parasite from the tissues of the host, and thus preserve them from further destruction, may be regarded as beneficial. Investing sheaths similar in appearance, though somewhat different in origin, are well known in the case of Tachinidae, where they arise in part at least as the result of mechanical injury to the hypodermis or tracheal epithelium of the host. In the case of polyembryonic hymenopterous parasites, an investing sheath is not uncommon and Marchal has figured a delicate membrane enveloping the larva of *Platygaster* which appears to arise by proliferation of the basement membrane of the host. Hollande (1920) has described a similar sheath formed from the peritoneal membrane of *Dytiscus* and surrounding cysts of *Distomum*. But such sheaths are very different from that found in *Saissetia* parasitised by *Encyrtus*, since the nuclei are persistent throughout and the cellular nature of the membrane easily seen. It is the apparent absence of mechanical stimulation of the tracheal system and the conspicuous part played by phagocytes in the formation of the sheath surrounding *Encyrtus* which is peculiar. The only process at all closely resembling it of which I have been able to find any published account is described by Lartschenko (1933) in a paper on the "non-susceptibility" of the larvae of *Loxostege* and *Pieris* to attack by *Anilastus* and other hymenopterous parasites. This author describes capsules formed around the eggs and larvae of the parasite by the "mesenchyme cells" of the host. The capsule is many cells thick but the innermost cells quickly form a syncytium whereupon their nuclei break up and disappear. The cells themselves then quickly degenerate to form a more or less homogeneous connective tissue. As time goes on, the inner layer of the capsule thickens at the expense of the outer, a layer of cells in an intermediate transition stage being visible between. Lartschenko criticises Lazarenko on the ground that he fails to distinguish between phagocytes and "mesenchyme cells", but it appears to the present writer that the former's method of dis-


F.B.H.
Ph.
T_1
M.H.
F.B.P.
O.
C.P.
15
0·025 mm.
T_2
T_1
Sh.
F.P.
T.A.
0·25 mm.
17
Sh.
Ph.
T.A.
C.
C.P.
T_1
F.P.
F.B.
16
0·1 mm.
0·5 mm.
18

Figs. 15–18.

tinction is far from convincing and Lazarenko's interpretation seems preferable.

It is remarkable that the peculiar phagocytic action described above, which results in sheath formation, seldom starts before the larva reaches the fourth instar. Previous to this the phagocytes react only to dead or moribund eggs or larvae and their reaction is of the normal type shown in Fig. 1, in which a dense cellular envelope several layers thick is formed. What can it be which determines this specific phagocytic action in response to the fourth-instar larva and not before? It is possible that some change in the metabolic rate of the parasite might be responsible, although if this were so one might expect this type of sheath to be found frequently in the case of parasitised insects. It is perhaps more likely that the sheath is developed in response to some secretion produced by the segmental dermal glands. These glands are possessed by the fourth- and fifth-instar larva, but are not noticeable earlier. They consist of six large patches of specialised hypodermal cells situated in the pleural region of segments 2–7. They cause a slight pad-like projection on the body wall, and are very easily seen if the living larva is placed in a solution of methylene blue (Fig. 18), which stain they take strongly, the rest of the body remaining uncoloured. The histology of these hypodermal glands is shown in Fig. 19. I have been unable to determine for certain whether the cuticle covering the gland is perforated or not, but there is certainly definite appearance of glandular pores when seen under an oil immersion lens. When these patches were first observed it was thought that they might be actually responsible for the secretion of the sheath, but this is clearly not the case. Since the substance of which the sheath is composed stains well in sections treated with Mann's methyl blue-eosin secretory activity of this sort should be easy to demonstrate if it were in fact occurring.

(2) *Histology of healthy* Saissetia *in relation to sheath formation*

A study of the histology of a healthy scale insect reveals certain facts which seem very significant in connection with the formation of a sheath. Fig. 20 shows a section through a healthy second-instar scale insect and it will be seen that peritoneal membranes are developed to an unusual degree. Not only are such membranes around the various organs conspicuous, but

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Text to figs. 15–18.

Fig. 15. Portion of a transverse section of a scale insect containing a third-instar parasite larva showing phagocytes commencing to form a membrane sheathing the parasite. Mann's methyl-blue eosin.

Fig. 16. Portion of a transverse section through host containing a parasite in the pupal stage. The section passes through a tracheal attachment and a faecal pellet. Mann's methyl-blue eosin.

Fig. 17. Transverse section through a pupa dissected out from the host with its sheath intact. The section passes through the posterior tracheal attachments and a pair of the faecal pellets.

Fig. 18. Living larva (fourth instar) stained in methylene blue in order to show segmental glandular patches.

there is also a remarkable "supravisceral" or peritoneal membrane; in structure and staining properties very similar to the sheath investing the parasite larva. A study of sections of young scale insects shows clearly that this membrane is, in fact, produced by phagocytic action as described by Lazarenko (see above, Fig. 15), and it is also interesting to note that fine tracheal branches are frequently observed running over the surfaces of the membrane and in places actually passing through it (Fig. 21). We may say then, that the development of a sheath around the parasite consists simply of an augmentation and adaptation of a process which is normal to the healthy insect. When the parasite larva changes its position it comes to lie under this supravisceral membrane and this perhaps accounts for the greater thickness and pigmentation of the dorsal part of the sheath. The pigmentation is probably due to a setting free of a tyrosine or other chromogen by the degenerating phagocytic cells or possibly by the parasite itself; the chromogen then being acted upon by the tyrosinase of the host in the manner suggested by Ries (1932).

As mentioned above, the production of such a sheath may be highly beneficial to the host provided it develops early enough to save tissues from infection and destruction. In the case under discussion it develops so late that it has probably lost any such protective value it might otherwise have had; unless it can be considered as a protection against faecal contamination. Were it not for the development of the tracheal attachments it might even at the eleventh hour have saved the host by suffocating the parasite. As it is, the tracheal attachments prevent this and the sheath ultimately does nothing more than provide the parasite with a chamber for pupation. It is as if the final effort of the host is neatly utilised by the parasite for its own ends.

(3) *Significance of the tracheal attachments*

The origin and significance of tracheal attachments are far more difficult of explanation. What are the alternatives?

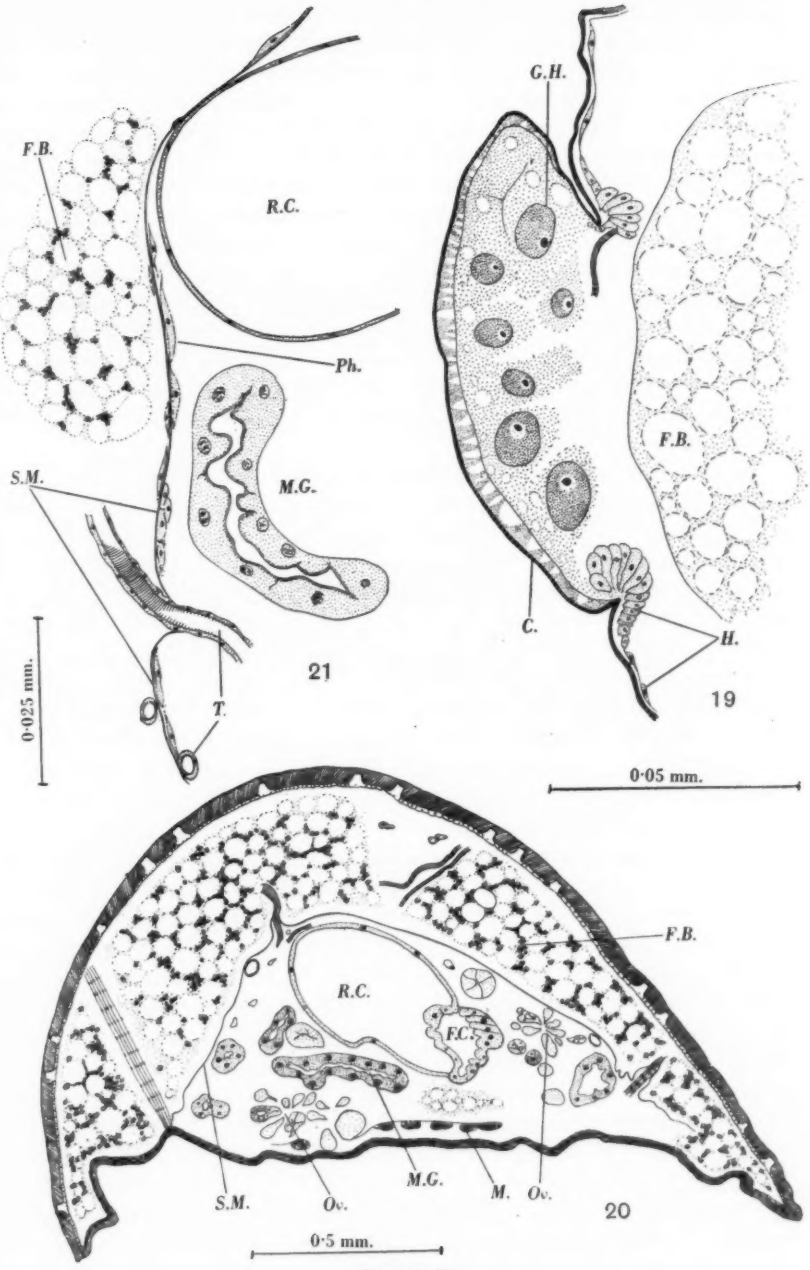
One view might be that these attachments are of no value to the parasite and are not in any sense an adaptation. There are many instances of "adaptations" so-called which will not bear critical examination. But although such an explanation is possible, it appears exceedingly unlikely. It cannot be just chance that the attachments develop as they do; just chance that they come opposite the spiracles of the larva. Such a linkage is not a normal reaction of tracheae to the tissues of the animal itself any more than to those of a parasite. To explain a linking on of this nature it seems necessary to postulate some special inducing mechanism on the part of the parasite.

Text to figs. 19–21

Fig. 19. Transverse section of a portion of the body wall of a fourth-instar larva to show histology of glandular area of hypodermis.

Fig. 20. Transverse section through a healthy scale insect in second instar to show "supravisceral" connective tissue membrane.

Fig. 21. Section to show formation and structure of "supra-visceral" membrane in healthy scale insect.


F.B.
R.C.
Ph.
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0·025 mm.
21
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19
0·05 mm.
F.B.
R.C.
F.C.
S.M.
Ov.
M.G.
M.
Ov.
20
0·5 mm.

Figs. 19–21.

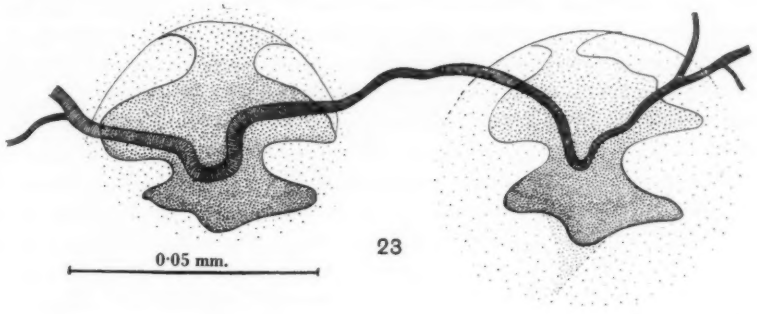
That the attachments are of value to the host seems entirely ruled out. In contrast to the process of sheath development it does not seem that the tracheal attachments can, under any circumstances or in any stage of development, be regarded as "useful" to the scale insect. In view of the experiments with oil, mentioned above, the only reasonable conclusion seems to be that the arrangement is actually of advantage to the contained larva.

If then the development of the tracheal attachments is stimulated by the parasite, what possible mechanism can be visualised? In the first place, can there be some mechanical injury to the tracheal trunks at these four or six points, which results in a sudden activation of the tracheal epithelium. This explanation seems to be excluded by the fact that there are no projections or roughened areas on the surface of the larva which could cause such an injury. The only means for injuring the host tissues which the larva has at its disposal are the mandibles, and it seems quite impossible to suppose that the larva can turn and puncture the tracheae with exact precision at these four or six points.

If, therefore, a mechanical explanation cannot be formulated, perhaps a physiological explanation is possible. Is the parasite secreting some substance which stimulates the tracheal epithelium at these critical points? In this connection it is worth mentioning that the points on the tracheal trunks which give rise to the attachments do not appear to be morphologically peculiar in any way. It is not as if they were regions where profuse branching normally occurs, or where the tracheal epithelium in the healthy insect shows any particular sign of activity. For this reason it would seem that any substance which is being liberated by the parasite must be set free only in the immediate neighbourhood of the future tracheal attachments rather than at random into the body cavity. It seems impossible therefore to regard the segmentally arranged dermal glands, above referred to, as being responsible for the activation of the host tissues.

Since the attachments occur at points on the sheath near to, but not coincident with, the underlying open spiracles of the parasite, the suggestion presents itself that the activation must be due to some substance diffusing out from the body of the larva in the immediate neighbourhood of the spiracles. But sections fail to give any indication of spiracular glands which could be held responsible. The only reasonable explanation seems to be that the tracheal epithelium of the host is activated by some sudden change in respiratory activity, a lowering of the oxygen tension or a great increase in the carbon dioxide concentration consequent upon the approach of the pupal stage of the parasite. It is reasonable to suppose that such a change in concentration would be felt most strongly opposite the open spiracles. Especially would this be so if, as seems to be actually the case, a small bubble of gas is extruded from the spiracle at about this time. One can argue not unreasonably that the tracheal epithelium would be particularly sensitive to a change of this nature, and evidence is not altogether lacking that some such mechanism

governs the development of the tracheal supply of rapidly growing organs. Fine tracheal branches have already taken part in sheath formation and the fusion of the main tracheal trunks with the sheath in this manner is perhaps not such a very big step further. Experiments are being planned which, it is hoped, may throw more light on the response of a tracheal system to such changes.



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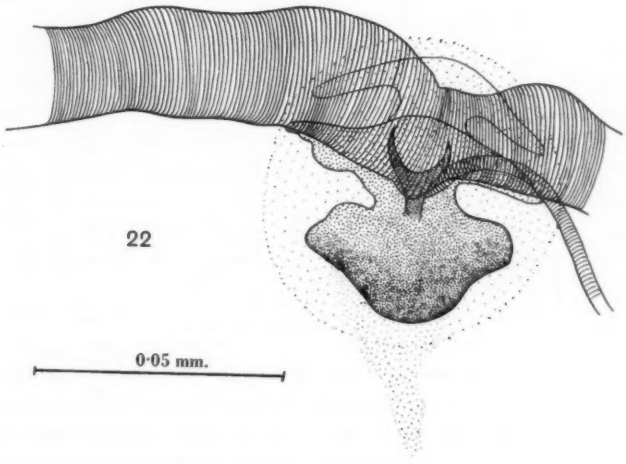

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Fig. 22. Posterior tracheal attachment as seen in "whole mount" of a sheath which has been dissected off. (Faure's fluid.)

Fig. 23. Double anterior tracheal attachment as seen in "whole mount" of a sheath, which has been dissected off. (Faure's fluid.)

The view that the attachment of the tracheal trunks to the sheath is indeed brought about by physiological rather than mechanical means receives strong support from a close study of serial sections. Although the formation of the thickened patches on the surface of the sheath is apparently the result

of phagocytic activity, it is obvious that the tracheal epithelium is also activated in some way, for it will be seen from Figs. 22 and 23 that the trachea becomes very sharply bent inwards at the point of attachment. In some cases (see Figs. 23 and 24) there is almost a right-angled bend, and this bend in many, if not in all, cases results in an actual fracture of the tracheal lining. The shape and dimensions of the bend are such that it is impossible to suppose that it can have been produced by mechanical stress after the attachment has taken place. It seems certain that the kink must have been produced by a sudden growth of the cells of the tracheal epithelium on one side of the trachea only: a development such as would be brought about by the diffusion outwards of some activating substance from a localised area upon the surface of the sheath.

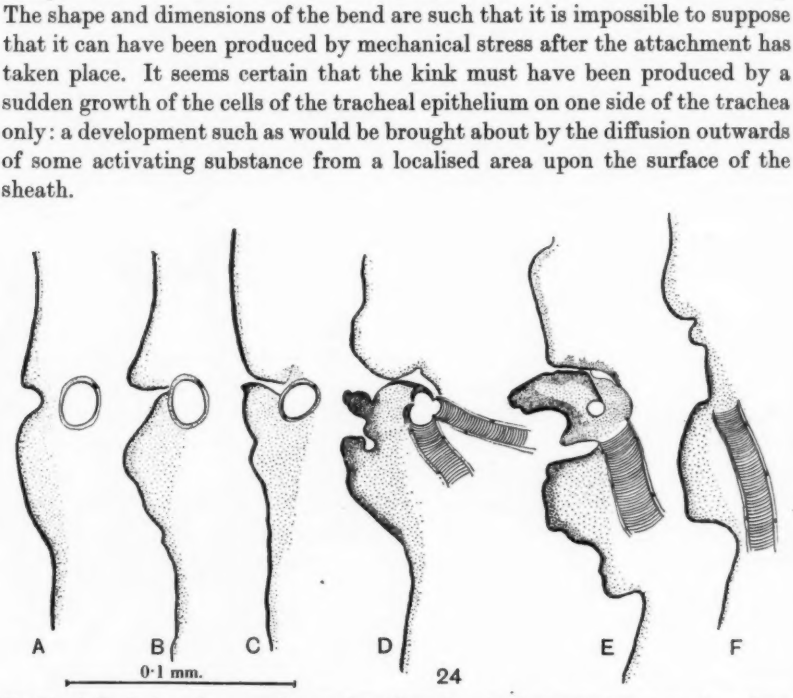

Fig. 24. A–F. Series of consecutive sections through a tracheal attachment to show sharp bending of the host trachea.

It should be mentioned that experiments in which *Polytoma* cultures were used as indicators of oxygen consumption (Thorpe, 1932) were carried out on larva and pupae which had been dissected out from the hosts intact in their sheaths. These experiments gave negative results, and no particular localised response of the *Polytoma* cultures to the spiracular regions could be detected, but this lack of a result may be explained by the fact that the spiracular areas are so small that in this case it is hardly to be expected that the method would give reliable results.

IV. "Puparia" and sheath formation in other chalcid parasites

In conclusion it seems convenient to summarise very briefly work which has been done on some other chalcid parasites closely related to *Encyrtus infelix*. The work of Embleton, to whom is due the credit for first observing

various peculiar features in the biology of *Encyrtus*, has already been mentioned, and does not require further discussion.

In 1918 Imms gave an account of *Blastothrix sericea* and *Aphycus melanostomatus* parasites of *Eulecanium coryli* (*Lecanium capreae*), the last larval stages of which become enclosed in a "sheath-like investment" covering the entire body except the head and which is intimately connected with the tracheal system of the host. Imms concluded that the sheath in these cases was formed entirely by the host, and he suggested that it might, as in the Tachinidae, arise as a proliferation of the tracheal epithelium, probably as a result of injury or perforation by the parasite. He figures quite large tracheal branches opening directly into the sheath, but they differ from those of *Encyrtus infelix* in being quite irregular in position. In some cases the sheath is apparently lacking altogether.[1] Dr Imms has very kindly allowed me to examine a number of his sections of this parasite, and it is clear that the sheath is very similar in structure to that of *E. infelix* and almost certainly arises in the same way. Imms observed a large number of conidia-like bodies enclosed in the matrix of the sheath identical with the symbionts present in the hosts' fat body. Such conidia, although numerous in the fat body of *S. hemisphaerica*, are much less obvious in the sheath of *Encyrtus* and do not appear to have any connection with its formation. They are apparently liable to escape into the haemocoele as the fat body is destroyed by the feeding of the larva and their abundance on the outer surface of the sheath may depend on the quantity of fat body which has been destroyed.

Timberlake (1913) and Smith & Compere (1926) have described the pupa of *Coccophagus* as being "enclosed within a puparium", which they state is derived from a persistent larval skin. They make no mention of any tracheal connections. These species are also parasitic on Lecaniine scale insects of the genera *Coccus*, *Lecanium* and *Saissetia*.

In 1919 Silvestri described *Encyrtus infidus* (Rossi), a parasite of *Sphaerolecanium prunastri*. The structure and habits of the early stages of this insect are very similar to those of *Encyrtus infelix*, but the author makes no mention of any turning movements of the parasite, nor of any investing sheath or tracheal attachments, although he does mention a darkened area over the dorsal region of the fourth-stage larva, which he suggests may consist of a secretion of the lateral dermal glands.

In 1932 Clausen gave an account of the biology of a species occurring in Chosen (Korea) which had also been determined as *Encyrtus infidus* (Rossi), a parasite of *Lecanium kunoensis* (Kuw). It is, however, quite clearly different from that described by Silvestri. Clausen describes the last larval stage as being

[1] Since this paper went to press I have had the opportunity of examining a quantity of living material of *Lecanium capreae* parasitised by *Aphycus melanostomatus* in which the sheath was never fully developed and was usually entirely lacking. This is probably to be accounted for by the fact that the host belonged exclusively to the first generation and were so small that the tissues had been almost completely destroyed by the feeding of the parasite. When the larger second generation scales are attacked a sheath is usually formed.

surrounded by a sheath, and he also describes and figures the attachment of host tracheae somewhat similar to those found in *E. infelix*, but he does not go into details nor does he make any suggestion as to the means whereby such attachments could be brought about. Clausen considers that the sheath is a true puparium composed primarily, if not entirely, of the last larval skin, for he apparently failed to observe a cast skin lying within the sheath at the hind-end of the pupa.[1] His suggestion that the sheath is a larval skin is the natural one considering that he did not cut sections. Without section cutting it is impossible to determine its true nature. There seems little doubt that the biology of the *E. infidus* found in Chosen is very closely similar to that of *E. infelix*. I am inclined to think that all cases of so-called puparia found among chalcid parasites of Coccidae will prove to be due to sheath development on the part of the host.

Finally, there is the remarkable case of the encyrtid *Carabunia myersi*, a very brief account of which has been given by Myers (1930). This insect is a parasite of froghopper nymphs (*Clastoptera undulata*) in Cuba. In this insect the egg lies freely in the body cavity and the early larval stages, although of the caudate form, are without connection with the atmospheric air. In the last larval stage a sheath together with tracheal attachments, apparently very similar to those of *Encyrtus infelix*, is formed. Here again the sheath is naturally described as a puparium, and three pairs of tracheal attachments are described. Unfortunately, since the author was engaged in economic work which involved constant travel, he was unable to investigate the matter adequately, and his account, while interesting, is so slight that it is impossible to know how far the case is comparable to that of *Encyrtus*. It is obviously an example of extreme interest, which, as the author himself says, would well repay really careful investigation.

V. Summary

1. The life history of *Encyrtus* (*Comys*) *infelix* (Embleton), a hymenopterous parasite belonging to the chalcid family Encyrtidae, is described. It parasitises *Saissetia hemisphaerica*, a member of the subfamily Lecaniinae (Homoptera Coccidae). Its respiratory relationships with the host are of a quite extraordinary character.

2. The egg is provided with a hollow stalk which is left protruding from the posterior dorsal body wall of the host. The first three larval instars are metapneustic, the spiracles being placed at the tip of a pair of long caudal processes which are inserted in the hollow stalk. It has been proved that these spiracles are open and in actual communication with the atmospheric air, which enters through the pedicel.

3. The fourth and fifth larval instars are amphipneustic and the manner

[1] I am much indebted to Dr Clausen for his kindness in sending me preserved material of *E. infidus*. The last larval skin can be found within the sheath just as in *E. infelix* but is much more difficult to see owing to an accumulation of waxy matter around it.

of respiration is entirely changed. The caudal processes degenerate and finally break away. The fourth instar larva turns round in the scale insect and becomes invested with a closely fitting transparent membraneous sheath produced by the phagocytes and fine tracheal branches of the host. This process appears to be merely a special case of the type of phagocytic activity which normally gives rise to the basement membranes and "connective tissue" membranes; such membranes are particularly conspicuous and well developed in *Saissetia*. The sheath becomes attached in an extraordinary manner to the main lateral tracheal trunks of the host in four (or six) places in the neighbourhood of the larval spiracles. This process is described in detail. It has been proved by an injection technique that an actual connection is established between the lumen of the host trachea and the cavity of the sheath. Bubbles of gas appear inside the sheath close to the points of attachment and near the spiracles of the larva which are thus put into functional communication with the tracheal system of the host.

4. While the production of the sheath might under certain circumstances be of value to the host the development of tracheal attachments appears to be of value to the parasite alone. The conclusion that the whole structure is an adaptation for the respiration of the parasite seems inescapable. A theory is tentatively put forward to account for the stimulation of the host tracheal epithelium by a sudden physiological change in the tension of the respiratory gases in the region of the parasite spiracles.

5. References to similar instances among the Chalcids are briefly reviewed. It is suggested that the "puparia" which have been described in the case of one or two other Chalcidoidea may be found to arise in the same way.

In conclusion I wish to express my gratitude to Dr A. D. Imms, F.R.S., and Prof. D. Keilin, F.R.S., for their helpful and stimulating interest in this investigation. To Dr Imms I am particularly indebted for allowing me to examine his sections of *Aphycus melanostomatus*.

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Lettering of Figures

C. cuticle.
C.P. cuticle of parasite.
C.Pr. caudal process.
C.S. cast skin.
D.T.C. dorsal transverse commissure.
E. egg shell.
F.B. fat body.
F.B.H. fat body of host.
F.B.P. fat body of parasite.
F.C. filter chamber.
F.P. faecal pellet.
G.H. glandular region of hypodermis.
H. hypodermis.
Lb. labial region.
Lr. labral strut.
L.T. lateral tracheal trunk.
M. muscle.
Md. mandible.
Md.C. mandibular condyle.
M.G. mid gut.
M.H. muscle of host.
M.T. Malpighian tube.
Mx. maxillary regions.
O. oenocyte.
Ov. ovary.
P. protoplasmic plug in egg stalk.
Ph. phagocytes.
R.C. rectal chamber.
S. spiracle.
S.A. anterior spiracle.
Sh. sheath.
S.Ht. spiracle of host.
S.M. supra visceral membrane.
S.P. posterior spiracle.
T_1 trachea of host.
T_2 trachea of parasite.
T.A. tracheal attachment.
T.C. tracheal commissure.
V. vestibule of spiracle.
V.T.C. ventral transverse commissure.

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RESEARCHES ON THE INTESTINAL PROTOZOA OF MONKEYS AND MAN

VIII. AN EXPERIMENTAL STUDY OF SOME SIMIAN STRAINS OF *"ENTAMOEBA COLI"*

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There is a considerable amount of difficulty and numerous sources of fallacy to be encountered in proceeding to the consideration of *Amoebae*.
—D. D. Cunningham (1871, p. 190).

CONTENTS

	PAGE
I. Preface	541
II. Materials, Methods, and Preliminary Observations	545
(1) Experimental animals	545
(2) Notes on natural infections	547
(3) Cultivation of *E. coli*	550
(4) Strains of "*E. coli*" studied	555
III. Experiments and Results	558
(1) The longevity of "*E. coli*" cysts	558
(2) Infection of man with *E. coli macacorum*	563
(3) Infection of *Macacus sinicus* with *E. c. jacchi*	566
(4) Infection of *M. rhesus* with *E. c. jacchi*	568
(5) Infection of man with *E. c. jacchi* after passage through *M. sinicus*	570
(6) Duration of infections	573
(7) Attempts to infect kittens	575
(8) Ingestion of red blood-corpuscles by *E. coli*	577
IV. Discussion	580
Summary and Conclusions	590
References	591

I. Preface

In the ensuing pages I shall describe and discuss various experiments made during the last dozen years with the object of elucidating the natural history of the large harmless intestinal amoeba of Man commonly called *Entamoeba coli*.[1] Little mention has been made of this organism in previous instalments of these *Researches*,[2] though it has claimed my attention continuously from the beginning.

[1] A detailed summary of all the earlier work on this species, with full references, will be found in my monograph (Dobell, 1919).

[2] See Dobell (1928, 1929 [with Bishop], 1931, 1933, 1934, 1935): also the annual *Reports of the Medical Research Council* for 1924–5 to 1934–5.

Readers (if any) of foregoing parts of this series will be familiar by now with my programme of work and its general aims: but as further explanation is necessary before giving the present results in detail, I shall here note with the utmost brevity—by way of introduction—a few particulars essential for the understanding of all that follows immediately.

I have attempted to unravel the tangled and hitherto largely unknown life-cycle of *E. coli* by studying not only this well-known species (in Man), but also by utilizing the comparable amoebae which inhabit various monkeys. As yet, however, I have had at my disposal only 3 living and naturally infected simian subjects—2 macaques (*Macacus sinicus* and *M. rhesus*) and 1 marmoset (*Hapale jacchus*). Present knowledge of the spontaneous infections in these hosts is briefly as follows:

(i) When I began these researches in 1924 it was already well known that macaques of at least 3 species—possibly more—are sometimes naturally infected with amoebae closely resembling *E. coli* (of Man), and since then several others have been found to harbour similar protozoa. It was not—nor is it even now—certainly known, however, whether all these amoebae, in different macaques, belong to one species; and whether or no any or all of them can properly be assigned to *E. coli*. The best-known form was that occurring in *M. rhesus*; but this had been recorded by Mathis (1913) as a distinct species (*E. legeri*), which was described later by Mathis and Mercier (1917) in some detail. Most other records are still scanty and inexact, and many published findings are hard to interpret; but so far as I have been able to ascertain "*E. coli*" (or "*E. legeri*") has now been found in no less than 9 different species of *Macacus*—as shown in the following list:

Species of *Macacus* [=*Macaca*=*Silenus*]	Recorder
(1) *M. sinicus* [=*radiatus*]	Brumpt (1909)
(2) *M. rhesus* [=*mulatta*]	Mathis (1913)
(3) *M. tcheliensis*[1]	Mathis (1913)
(4) *M. cyclopis*	Morishita and Tsuchimochi (1926)
(5) *M. irus* [=*cynomolgus*]	Kessel (1928)
(6) *M. sancti-johannis*	Kessel (1928)
(7) *M. lasiotis*	Kessel (1928)
(8) *M. nemestrinus*	Dobell (1928)
(9) *M. philippinensis*	Hegner and Chu (1930)

This list is probably incomplete, and gives the name of the first recorder only (in so far as I can determine it). "*E. coli*" has been noted, however, in several of these hosts by other workers: for example, in *M. sinicus* also by Dobell and Laidlaw (1926*a*), Deschiens (1927), and perhaps by Mello (1923); and in *M. rhesus* by Dobell (1919, 1928), ? Mello (1923), Bishop (1927), and Kessel (1928). Nevertheless, these few data indicate clearly that amoebae resembling *E. coli* must be widely distributed among the Macaques generally. I have personally studied the forms inhabiting *M. sinicus*, *M. rhesus*, and *M. nemestrinus* only.

[1] By some systematists regarded as a subspecies of *M. lasiotis*.

(ii) Very little is yet known about the intestinal protozoa of the Marmosets, and so far as I am aware there is still no published record of amoebae from this group. The only information which I have been able to collect from the literature is a statement by Faust (1931) that he examined "marmosets [number and species not recorded]" in Panama, and found them "negative for endamoebae": and a note by Hegner (1935) mentioning that he studied 5 marmosets (*Leontocebus geoffroyi*)[1] in the same locality and found "2 negative; 3 with *Trichomonas*"—therefore, presumably, none infected with amoebae. Recently Rodhain (1933) has recorded a new coccidian (*Isospora arctopitheci*) from *Hapale jacchus*, but he likewise makes no mention of finding amoebae in this host. Consequently, I believe that the "*E. coli*" which I have found in a marmoset, and have studied in detail, has not been observed previously.

Amoebae resembling *E. coli* have been reported[2] not only from the Macaques but also from the Anthropoid Apes (gorilla, orang-utan, and chimpanzee) and various other Primates—both catarrhine and platyrrhine. For the moment I shall say nothing about any of these, as they lie beyond the bounds of my present inquiries. I have purposely confined myself within very narrow limits—my object, in all these researches, being to gain a little exact knowledge rather than much that is uncertain. Accordingly, the following pages deal only with *E. coli* (of Man) and the similar species occurring in the Macaques and a single Marmoset. All other comparable—possibly identical—amoebae in other hosts will be left out of account for the present.

I have already expressed the opinion[3] that the *coli*-like species of *Entamoeba* naturally inhabiting the Macaques is probably *E. coli* itself: for on morphological grounds it seems impossible to distinguish it from the species in Man. I must now add that I have drawn the same conclusion regarding the similar amoeba which I have found in a marmoset. Some experimental evidence in support of these views will be given in the following pages; but to make my terminology clear—without prejudice to the issue (which has already been partially prejudged)—I must here explain the nomenclature now adopted.

Entamoeba coli here denotes the species of human intestinal amoeba described and defined under this name in my monograph (Dobell, 1919). When I have to distinguish it from similar—possibly or probably or even certainly identical—amoebae in other hosts, I call it *E. coli hominis*. The closely comparable (? identical) amoebae naturally living in a macaque or a

[1] It was one of these animals, presumably, which Hegner, Johnson, and Stabler (1932) claim to have infected experimentally with *E. histolytica*. Of this experiment I shall say more anon.

[2] Some writers refer all simian amoebae resembling *E. coli* to this species, while others regard forms found in different primate hosts as distinct. At present there is little scientific justification for either assumption. "*E. coli*" of "monkeys" generally is sometimes called *E. pitheci* (Prowazek, 1912): cf. Dobell (1919).

[3] Dobell (1928, p. 362), *et alibi*.

marmoset I designate *E. coli macacorum* or *E. coli jacchi*, respectively,[1] whenever it is necessary to discriminate between the forms in these several hosts—though I now believe them all to be specifically the same. I do not propose the epithets *hominis*, *macacorum*, and *jacchi* as varietal or subspecific designations under the International Code, but use them merely as self-explanatory terms for convenience of description: and when I cannot, in argument, legitimately assume the specific identity of the amoebae in men, macaques, and marmosets, I call them all "*E. coli*" (in inverted commas) to indicate the element of doubt—doubt in the reader's mind, but no longer in mine. In our present state of ignorance it is difficult—often impossible—to determine the "correct" names of these amoebae.

As in all other experiments made in the course of this series of investigations, I have devoted particular attention to the materials and animals employed. All my experiments have been made with pure strains of amoebae—carefully isolated and studied *in vitro*—and with animals proved, as far as possible, to be uninfected before inoculation. Furthermore, every experiment has been followed up for a time sufficiently prolonged to demonstrate its outcome with certainty. If the reader should think that my precautions have been excessive, and that my insistence on these details is unnecessary, I would remind him of some other results which have been published during the progress of the present work. After I had proved that *E. histolytica* is transmissible to macaques, three other workers[2] attempted to show that this amoeba can also parasitize several New-World monkeys. But as no adequate examination of any animal appears to have been made before inoculation, the subsequent findings reported cannot be accepted with any assurance. For example, among the monkeys which these workers claim to have infected there is a marmoset (No. 173)[3] into which faecal material containing *E. histolytica* was injected. Only one "negative" examination of the marmoset's own faeces appears to have been made previously—on the day of the experiment: and as evidence of success it is recorded that at necropsy, 20 days later, "amoebae containing red cells were found in the cecum".

Now to a casual reader this "experiment" may appear instructive: it might even seem to increase our knowledge by proving that *E. histolytica* is transmissible to marmosets. But to me it appears merely obstructive; because it actually proves nothing, and even adds confusion to a subject already sufficiently complicated. No interpretation of this experiment is really possible until it has been repeated with suitable safeguards. In support of this view I shall show in the present paper—though it is almost axiomatic—that a single "negative examination" is worthless as evidence of non-infection in

[1] I use the generic genitive plural for the former and the specific genitive singular for the latter because I find no reason to suppose that there is more than one "*E. coli*" in *Macacus* generally, while I have as yet no general information about marmosets—having studied only a single individual of one species (*H. jacchus*).

[2] See Hegner, Johnson, and Stabler (1932).

[3] *Op. cit.* p. 423.

a marmoset; and further, that a marmoset may be naturally infected with entamoebae which can ingest red corpuscles. Moreover, the haematophagous amoebae which I have found in this host have turned out to be not *E. histolytica* but *E. coli*.

Consequently, while I have no doubt that my American co-workers made and recorded their experiments in all good faith, and with a genuine desire to advance our knowledge, I am none the less convinced that their methods are fallacious and their conclusions correspondingly unacceptable. I am confident that my own more pedestrian procedure—demanding adequate control at every step—is scientifically sounder, and therefore more likely to yield results of permanent value.

The main object of the present work was to discover the unknown metacystic stages in the life-history of *Entamoeba coli*. In this endeavour I have now succeeded—more or less—and I hope to publish my findings in the next instalment. But a secondary aim was to ascertain, by experiment, whether or no the "*E. coli*" which naturally inhabits various monkeys really belongs to this species. Part of my evidence for answering this question affirmatively depends upon the observed development of several strains of amoebae *in vitro*: the other part is based upon the results of a few attempts to transmit divers strains from one host to another. It is this aspect of the problem which is here described and discussed particularly. For the investigation of every branch of the present inquiry, however, the acquisition of pure strains of amoebae—cultivable continuously *in vitro* at all stages—has been an essential preliminary. Accordingly, I have paid particular attention to this subject; and I therefore now record various experiments connected with cultivation, encystation and excystation, the viability of cysts, and the behaviour of amoebae, with a degree of detail which may at first sight seem superfluous. The importance of these details will, I hope, become self-evident in the sequel.

As a motto for the present paper I have borrowed a few words from a distinguished naturalist and pioneer in Tropical Medicine who was one of the first—perhaps the very first—to study *Entamoeba coli*. His words seem particularly appropriate, as they were used with special reference to this organism. Yet notwithstanding all his caution Cunningham himself unhappily fell into the traps against which he forewarned us, and I therefore repeat his admonition with some trepidation and a fervent prayer that I may be more fortunate. *Absit omen.*

II. MATERIALS, METHODS, AND PRELIMINARY OBSERVATIONS

(1) *Experimental Animals*

In earlier instalments I have already given some account of my general methods of work and my experimental animals, so I need merely note a few additional particulars here.

Though I obtained various strains of "*E. coli*" from several different simian hosts, I used only 2 macaques and myself for the present experiments.

The experimental monkeys were **Susanna** (*M. sinicus*=*radiatus*) and **Rosa** (*M. rhesus*=*mulatta*)—both of whom have been frequently mentioned in earlier descriptions of other investigations. These animals were normal and healthy in every way, and completely docile in my hands. They are still alive and well at the time of writing, and have now been in my possession for 12 years and 9 years respectively. Throughout these periods they have never been in contact with any other monkeys (or other animals of any sort) infected with *E. coli*.[1] Moreover, whenever I attempted to infect either experimentally, they were always carefully isolated from one another—though inhabiting the same room, in which they were separately caged—and have only been allowed to associate at times when they were known to be both infected or both uninfected. By exercising the strictest personal supervision I have been able, I believe, to exclude all chance of accidental infection during the whole period covered by these observations (about 12 years).

In the present researches I also used a few kittens, but further details regarding these animals will be noted later.[2] My general methods of keeping and inoculating kittens have already been noted elsewhere, in connexion with my experiments with *E. histolytica*, so I may refer the interested reader to what is there recorded.[3]

One other animal—of great importance for the present work—must be mentioned here, although it was used for no experiment and merely served as a source of material. This was a marmoset ("Willie"), in whose faeces I found an easily cultivable amoeba morphologically indistinguishable from *E. coli hominis*. As I have devoted special attention to this amoeba, which figures largely in the ensuing experiments, I add the following details about its host:

Willie (*Hapale* [=*Callithrix*] *jacchus* ♂) was one of a pair of Common Marmosets obtained from a dealer. They came to the Institute in September 1931, and were used by my colleague Dr J. R. Perdrau for some experiments with viruses. As these experiments were negative, and one marmoset afterwards died from "cage paralysis", Dr Perdrau kindly gave me the discarded survivor (Willie) in October 1932. At this date he was fully grown, but in rather poor condition. However, with careful feeding and personal attention he soon recovered, and he remained healthy and vigorous—though kept in strict isolation—while in my possession. I killed him in June 1934 after keeping him as a pet for over 19 months. During this time I made an exhaustive study of his intestinal amoebae (*E. coli jacchi*), which proved to be of great interest—as I hope to show presently.

I have every reason to believe that Willie's amoebic infection was natural, as he was never in contact—in the known period of his captivity—with any

[1] Before I infected myself with *E. coli* experimentally I did not harbour this amoeba: and the monkeys' only attendant (who cleans their cages daily, and occasionally prepares their food) was likewise uninfected—as I ascertained by microscopical and cultural examination of his stools. He never handles the monkeys, who will not allow anyone but myself to touch them.

[2] See p. 575 *infra*.

[3] See Dobell (1931), p. 10.

animal except another marmoset of his own species, or with any other known host of "*E. coli*".

(2) *Notes on Natural Infections*

I have made no attempt to study the incidence of "*E. coli*" in macaques or marmosets, or its distribution in monkeys generally. Nevertheless, during the present work I have collected a few data relating to simian infections, and these may be conveniently noted here.

As already recorded in previous reports,[1] my original family of tame macaques consisted of only 4 carefully examined individuals, to which I afterwards added 2 others (after ascertaining their natural intestinal infections with protozoa). These 6 monkeys were:

(1) **Polo** (*M. sinicus* ♂)
(2) **Jacko** (*M. rhesus* ♂)
(3) **Mungo** (*M. sinicus* ♂)
(4) **Susanna** (*M. sinicus* ♀)
(5) **Rosa** (*M. rhesus* ♀)
(6) **Bonar** (*M. sinicus* ♂)

Exhaustive examination of the faeces of all these animals revealed, curiously enough, that all the males were uninfected with "*E. coli*" but both the females infected. Susanna (*M. sinicus*) and Rosa (*M. rhesus*) were naturally infected, when they came into my possession, with a species of amoeba morphologically identical with *E. coli* (of Man)—the organism which, as already noted, I here call *E. coli macacorum* whenever it is necessary to distinguish it from *E. c. hominis*.

From time to time all the monkeys listed above were used for various experiments—already partly recorded—and in the course of these they were kept in strict isolation. But in the intervals between experiments they were often in the closest possible contact, so that their infections had every opportunity of spreading from one to another. I examined their faeces frequently during the years of their association, in order to follow individual infections and discover any possible spread; but, to my surprise, no uninfected male ever acquired infection with "*E. coli*" from his female companions. When, after several years, I killed off the males one by one—Bonar, Jacko, Polo, Mungo, in order—they were all still uninfected with "*E. coli*", though both females still harboured this species. These findings seem remarkable, and in view of certain other experiences[2] suggest that my originally uninfected male macaques were probably also uninfectible. But I made no attempts to infect any of them experimentally, so can offer no further evidence.

For several years I studied the natural infections in Susanna and Rosa, but finally they died out spontaneously in both. (The evidence for this statement will be given presently.) Susanna remained infected, however, for about

[1] See especially Dobell (1931), pp. 8–9.
[2] Especially with *Trichomonas* (Dobell, 1934) and *Enteromonas* (Dobell, 1935).

4½ years, and Rosa for about 2: and during these periods their infections were closely comparable with those commonly seen in Man. Amoebae or cysts were seldom present in their faeces abundantly, and often they were undiscoverable by microscopical examination. Negative examinations were frequently registered—sometimes consecutively over several months.[1] Positive findings were more often obtained by cultural examination, though even cultures made by the most appropriate methods often yielded negative results.

In order to illustrate the kind of findings which may be expected from a naturally infected macaque, I give herewith (Table I) the recorded results of the first 100 stool-examinations made on my monkey **Susanna**. These examinations were all made before any of the present experiments were performed, and are microscopical only—no cultural findings being entered. In the table, + means that amoebae or cysts (or both) of "*E. coli*" were found; − means that both were undiscoverable; and ? denotes that degenerate amoebae were present which could not be identified with certainty (the monkey being then also infected with *E. histolytica*—2 strains, large and small—and *E. nana*). The examinations were consecutive, though not made daily, and covered a period of 4 years (1924–1928).

Table I. *Results of* 100 *consecutive microscopical examinations of the faeces of* **Susanna** (M. sinicus ♀) *for* "E. coli"

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
+	+	+	−	−	?	?	−	−	+	?	?	−	−	−	−	−	−	?	−
21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
?	?	+	−	−	+	−	−	−	−	?	+	−	−	−	−	−	−	−	−
41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
−	−	−	−	−	−	+	−	+	+	+	−	−	−	−	−	−	−	−	−
61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
−	−	−	−	−	+	−	−	−	−	+	+	−	−	−	?	−	−	−	−
81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100*
+	?	−	+	?	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−

* The monkey was found still infected after the 100th examination was made.

It will be clear from this table that 17 of the 100 examinations were positive for "*E. coli*", 72 negative, and 11 doubtful: and it will be equally clear that—as in human infections with *E. coli*—a few negative examinations are worthless as evidence of non-infection in a macaque.[2]

[1] At such times the examinations were not made daily, but at intervals of several days or even weeks.

[2] Readers interested in this subject—the value of negative examinations in *E. coli* infections—will find a fuller discussion in an earlier report (Dobell, 1917): they may also be referred to the more recent work of Svensson (1935). As regards macaques, some valuable data were published long ago by Mathis (1913), who gave the results of 100 consecutive daily examinations of 4 several animals (all *M. rhesus*). Unfortunately, however, he did not treat *E. coli* (his "*Löschia legeri*") and *E. histolytica* (his "*L. duboscqi*") separately—his tables showing the findings for both species combined. Nevertheless, Mathis's records show that his 4 macaques (Nos. 4, 5, 6, 7) were "negative" for "*E. coli*" on 56, 32, 29, and 73 (out of 100) days respectively: and that long negative periods (19 consecutive daily examinations in No. 4, 26 in No. 7) may occur. All

This conclusion is supported also by the observations made on another infected animal—my marmoset, Willie. As already noted,[1] I studied him for over a year and a half; and as my findings during this period seem instructive, I shall record them very briefly here.

Willie (*Hapale jacchus* ♂) was examined altogether 78 times between 20. x. 32 (shortly after he came into my possession) and 2. vi. 34 (when I sacrificed him). All my examinations were made with great care, and were both microscopical and cultural—the method of cultivation being one which I soon found to be almost infallible for growing *E. c. jacchi*, whether present initially in the amoeboid or the encysted state. The marmoset usually passed faeces in the form of small and very hard pellets, resembling grape-seeds in appearance and consistency: and in such unpromising material I succeeded only once in discovering any amoebae microscopically, and only once (on another occasion) in finding a solitary cyst, in the course of making 62 examinations during the first year (20. x. 32–6. xi. 33). Less thorough microscopical investigation, therefore, would probably have failed to disclose any infection at all.

Cultural examinations, however, were more successful, for during the same period I recovered "*E. coli*"—usually in excellent and easily propagable cultures—on 9 different dates. The last positive culture was obtained on 6. xi. 33, and thereafter Willie's faeces were consistently negative—microscopically and culturally—for 6 months.

As the last positive examination had been preceded by a long series of negatives, and as I felt fairly certain that Willie's infection must have died out, I chloroformed him in June 1934 in order to make sure. At the necropsy I made a thorough microscopic search of his entire intestinal tract, and inoculated cultures with contents obtained at various levels (duodenum, caecum, colon, rectum). All the results of this laborious investigation were completely negative; and I was personally satisfied that, at his death, this marmoset was no longer infected.

My interpretation of all the findings is that Willie was infected with "*E. coli*" for about a year, though the infection was difficult to discover, and that it then died out spontaneously. I may add that he also apparently lost two other intestinal infections during the same period. When first examined, he was found to harbour not only "*E. coli*" but also a *Giardia*[2] and *Blastocystis*. Both these organisms vanished from his faeces a few months after I began to study him, and they were never afterwards discoverable.

examinations were, of course, microscopical only: and it should be noted that Mathis considered 3–4 minutes' examination sufficient to warrant (*affirmer*) a "negative" (*op. cit.*, p. 402). This is a standard which I know to be inadequate, and one far below my own. Indeed, the longer I live, the longer it takes me to make a "negative examination".

[1] See p. 546 *supra*.

[2] So far as I am aware, *Giardia* has never previously been recorded from marmosets of any species. In Willie I found only a few rare cysts—never flagellates, even in his duodenum *post mortem*—which differed from those of men and macaques by their broader and more rounded form.

I still do not know exactly how many negative examinations are necessary to prove that any animal is uninfected with *E. coli* (or any similar amoeba). I know only that a few negative examinations are worthless as evidence of non-infection, and that extreme care must be taken to disprove infection before any animal is pronounced "uninfected" for experimental purposes. For this reason I beg the reader to note the evidence of non-infection which I give in every experiment in the present series. I do not claim that it is absolutely conclusive, for I know that it is only—in a high degree—probable. I have numerous records which show that very many "negative examinations", over a long period, can be made on animals (including men and monkeys) which are really infected. As an instance, I may note that at one time I actually examined my marmoset (Willie) 45 times consecutively during a period of over 7 months (24. iii. 33 to 29. x. 33) with consistently negative results: in no microscopical or cultural examination could I then detect any infection with "*E. coli*". But at the 46th examination (6. xi. 33) I recovered this amoeba again in cultures of his faeces—proving that he had really been infected throughout.

Consequently, more than 45 consecutive negative examinations, made with every microscopical care and cultural control, may be needed to prove that a monkey is really uninfected with "*E. coli*". In my own work, I have never accepted less evidence as proof of non-infection; and I would naturally demand even more from other workers whose standards and methods of investigation appear to be less cautious and exacting.

(3) *Cultivation of* E. coli

In a paper published some 10 years ago (Dobell and Laidlaw, 1926*a*), it was shown that *E. coli* [*hominis* and *macacorum*] is cultivable by various methods similar to those devised by Boeck and Drbohlav for the cultivation of *E. histolytica*. We showed, moreover, that it was possible to obtain the complete life-cycle of this species *in vitro*, and by improving our original technique I have since been able to study every stage of development in detail. Several other workers have also been able to cultivate *E. coli* in recent years,[1] though not a few have failed.[2] *E. coli* is, indeed, more difficult to cultivate properly than any other amoeba living in Man; and to obtain pure cultures—free from all other species—in which active multiplication and encystation and excystation can all be obtained regularly and at will, is so difficult that nobody appears to have succeeded in the attempt hitherto.

Elsewhere[3] I have described my methods for studying *in vitro* the whole life-cycle of *E. histolytica*, and the same methods—with minor modifications—

[1] See especially Drbohlav (1925), Tanabe, Kuwabara, and Chiba (1930), Tanabe and Kuwabara (1931), Svensson (1935).

[2] Brug (1928), for example, mentions that he made 7 unsuccessful attempts to cultivate this species.

[3] See especially Dobell (1928, 1931).

are applicable to *E. coli*. Both species can be cultivated in various solid-liquid media of the Boeck-Drbohlav type, and both can be caused to encyst by the addition of solid rice-starch to such media. By keeping the amoebae growing in starch-free medium—in which they seldom form cysts—and then inoculating them into similar medium supplemented with starch, abundant cysts can often be obtained: and these cysts will then hatch—without any other treatment—and produce new cultures of amoebae on reincubation in fresh medium. This process can be repeated *ad libitum*.

But "pure" cultures of *E. coli* and *E. histolytica* grown in this manner are unavoidably accompanied by a complex bacterial flora; and it is the composition of this flora which largely determines success or failure. In the presence of certain bacteria the amoebae cannot even live: with others, they can multiply to an unlimited extent but cannot encyst: but when accompanied by the right bacteria they can not only multiply and encyst but also excyst, and so be made to go through their complete life-cycle over and over again—provided, of course, that attention is paid to the other factors (temperature, *p*H, etc.) necessary for their survival and development.[1]

Although I have devoted much time and work to the study of the bacteria necessary for the complete development of both *E. coli* and *E. histolytica*, I am still unable to describe or name them. I can still say only that I have obtained cultures which contain the "right bacteria" for each species (they are not the same), and that I hope to be able to give an adequate account of them later. The bacteriological analysis of such mixtures and the determination of the effects of individual micro-organisms on the growth of the amoebae are problems of great difficulty and complexity.

When culture-tubes of the Boeck-Drbohlav type are inoculated with faeces from a man or macaque infected with *E. coli*, they often give a good growth of this amoeba—mixed with other organisms—on incubation. The primary culture is frequently most promising. But when one attempts to continue the cultivation, and isolate a pure strain by inoculating subcultures, one seldom succeeds. In a few generations *E. coli* vanishes, and is replaced by other protozoa and *Blastocystis*. To obtain a pure strain of *E. coli*, free from all other protozoa and *Blastocystis*, unaccompanied by starch-splitting and other unfavourable bacteria, but accompanied by those necessary for the complete development of this one species of amoeba, is very difficult indeed. Consequently, I failed in all my early attempts with this organism. I got nothing but mixed cultures or "pure" cultures containing only amoebae which I could not induce to encyst with any regularity (because the proper bacteria were lacking). This was my experience, at first, with all strains of *E. coli* from Man.

With *E. coli macacorum* I had even less success initially. My two macaques which harboured this species were also infected with *E. histolytica*, *E. nana*, and flagellates—as well as *Blastocystis* and starch-splitting bacteria. To isolate

[1] The statements in this paragraph are supported by very numerous experiments which are, for the most part, still unpublished.

E. coli from such a mixture seemed almost impossible, as all the media employed were found to be more favourable for the cultivation of the other species. On many occasions, however, I succeeded in obtaining excellent cultures containing only *E. coli* and *E. histolytica*: but although it was often fairly easy to separate the latter in pure culture from this mixture, I could never isolate the former. Indeed, I have almost always found that when these two species are cultivated together, in any of the usual media, *E. histolytica* alone ultimately survives. After propagation for a variable number of cultural generations, *E. coli* generally dies out completely, leaving a pure strain of *E. histolytica*.

I made many attempts to obtain pure cultures of *E. coli* by growing a mixed strain (*coli*+*histolytica*) in media containing emetine in various quantities. Since *E. histolytica* is extremely sensitive to this alkaloid, while *E. coli* is not,[1] it would appear easy to separate the latter in this way: but in reality it is extremely difficult. I have never succeeded in obtaining a pure strain of *E. c. macacorum* by the action of emetine on mixed cultures—in spite of numerous attempts.[2] The reason for this is that *E. coli* is usually so difficult to grow at all, when just separated from its host along with an antagonistic species and a fluctuating and partly unfavourable bacterial flora, that the introduction of any new factor (in this case emetine) which upsets the microbic equilibrium is apt to exterminate not only *E. histolytica* but most other protozoa as well (including *E. coli*). The true direct effect of emetine on *E. coli* can only be demonstrated, indeed, on pure strains which have become thoroughly adapted to life *in vitro*.

I finally succeeded in obtaining pure strains of "*E. coli*" from both of my naturally infected macaques (Rosa and Susanna) by treating the monkeys themselves with emetine, and thus removing their concomitant infections with *E. histolytica*.[3] When they were "cured" they passed "*E. coli*" alone in their faeces, so the chief source of difficulty was eliminated. By appropriate treatment of subsequent cultures—initiated from cysts, which I freed from other protozoa, *Blastocystis*, and starch-splitting bacteria by cooling, and treating with hydrochloric acid and acriflavine[4]—I got my first pure strain of *E. c. macacorum* (*RC.* from *M. rhesus*) in October 1928, and a second (*SC.* from *M. sinicus*) within a fortnight. Unfortunately, I lost the latter a few days later owing to the breakdown of an incubator in the night; and I was never able to replace it because the monkey (Susanna) unexpectedly lost her infection spontaneously about 2 months afterwards—before I had time to isolate another strain.

With Strain *RC.* (from Rosa, *M. rhesus*), however, I had more success.

[1] See Dobell and Laidlaw (1926) and Bishop (1929).

[2] My solitary success with this method was obtained later, when I was able—after many failures—to isolate Strain *MRC.* (*E. c. jacchi*) from Rosa (*M. rhesus*) at a time when she also harboured *E. histolytica* [*hominis*]. Both infections were, of course, experimental.

[3] See Dobell with Bishop (1929).

[4] See Dobell and Laidlaw (1926*a*) and Bishop (1929).

After making sure that it was uncontaminated with any other species of protozoon, I kept it going in starch-free medium ("Ehs") by daily transplantation. It grew excellently, and was undoubtedly pure: and when I transferred the amoebae to similar medium supplemented with rice-starch ("Ehs+S") they frequently produced good crops of typical 8-nucleate cysts on incubation. I studied this strain in detail, and used its cysts for numerous experiments (some of which will be described presently): but unfortunately it ceased to encyst regularly after several months, and so became useless for my purpose. I therefore abandoned it in March 1929, though I could easily have propagated it—growing excellently in the amoeboid state—for an unlimited period. I am now satisfied, as a result of further work, that Strain *RC.* lost its power to encyst through the dying out—on continuous cultivation—of certain necessary (though unknown) bacteria originally present.

I did not succeed in obtaining any other continuously cultivable and freely-encysting strain of "*E. coli*" until several years later (1932), when I began to study the marmoset (Willie) already mentioned. All my early attempts with *E. coli* [*hominis* and *macacorum*] failed, or succeeded only partially, but with the similar amoeba (*E. coli jacchi*) in *Hapale jacchus* I had complete success. Almost every strain—over a dozen—obtained from my marmoset was comparatively easily isolated, easily cultivated, and behaved consistently *in vitro* when properly treated. With a little coaxing at first, all the strains isolated from Willie's faeces grew well in "Ehs" medium, and could be propagated *ad libitum*. Moreover—and this was the important point—they all encysted regularly and freely when transferred into similar medium supplemented with rice-starch ("Ehs+S"); and the cysts so produced then hatched readily on incubation under suitable conditions. In other words, all my pure strains of *E. coli jacchi* behaved, in all essentials, like the earlier strains of *E. histolytica* which enabled me to study the complete life-cycle of this species *in vitro*.[1]

I have been able to prove that my strains of *E. coli jacchi* were so uniformly satisfactory for cultivation because they happened to be accompanied by the bacteria necessary for their complete development: for these bacteria (still incompletely studied, and as yet indefinable) when added to other non-encysting strains of "*E. coli*"—obtained from divers sources—convert them into strains which encyst and excyst as regularly as *E. c. jacchi*. My marmoset Willie thus supplied me not only with excellent material of "*E. coli*" for detailed study, but also with the bacteria which have enabled me to investigate many other strains of similar amoebae. For several years I have been able to isolate and study *in vitro*—at all stages of the life-cycle—the "*E. coli*" of any man or monkey whose infection I have wished to investigate. I can now, indeed, cultivate *E. coli*[2] almost as easily as *E. histolytica* from the faeces of any infected host, and can obtain any stage in its development at will.

[1] Cf. Dobell (1928).

[2] Provided, that is, that it is not accompanied by *E. histolytica*. Its separation from this species *in vitro* still presents great difficulties.

But although I have now solved the main problems of cultivation, for practical purposes, I am more than ever impressed with the difficulty of cultivating *E. coli*. The requirements of this species are exacting, and the continuous propagation of any strain *in vitro* demands ceaseless vigilance.[1] *E. coli* is very sensitive to even slight changes of temperature (optimum for cultivation 37°–38° C.), hydrogen-ion concentration (optimum pH = 7, with a well-tolerated range of 6·5 to 7·5), and other environmental factors; so that the continuous maintenance of even a single strain involves continual anxiety. It means frequent examination and transplantation of cultures, with strict attention to asepsis and control of all culture-media, and constant supervision of incubators during the entire period of cultivation. All strains must be cultivated in starch-free medium ("Ehs"), in the presence of suitable bacteria (uncontaminated with unfavourable organisms), in order that they may retain their properties and be made to form cysts when required by supplementing their diet with rice-starch: and under such conditions they grow well and remain normal only when the temperature and all other factors are kept constant.

Most strains when newly isolated and grown in starch-free medium have to be transplanted daily, though when well established they often grow excellently with subcultivation every other day. Occasionally cultures live for 3 days or even longer, but unfortunately they can never be depended on to do so.

The amoebae are so sensitive to cold that if the temperature of incubation falls to 34° C. they cease to develop and rapidly become abnormal. At lower temperatures they soon round up and die, while at ordinary room-temperatures (15°–20° C.) they can usually survive for only a few hours.[2] Consequently, the failure of an incubator for even a short period, or the accidental contamination of a culture-tube, may easily lead to the sudden loss of a valuable strain at any moment—as I well know from bitter experience.

Ripe cysts of *E. coli*, however, are able to live at low temperatures (1°–10° C.) for many weeks;[3] so it is a wise precaution, which I now invariably take, to obtain cysts from any cultivated strain at the earliest opportunity and keep a stock of these in cold storage—from which the strain can be recovered by hatching and further cultivation—in case of accidents.

[1] I refer, of course, to strains kept under proper control, so that any stage in development can be obtained at will at any moment. To keep a strain in the active amoeboid state (incapable of encystation) in starch-containing media is as easy as keeping a similar strain of *E. histolytica*: but such strains are of little value for experimental purposes, and useless for a study of the life-history.

[2] Starch-fed amoebae are much more resistant, and can sometimes survive exposure to room-temperatures for as long as 48 hours—as I have proved by experiments similar to those recorded for *E. histolytica* (Dobell, 1927) and *E. nana* (Dobell, 1933).

[3] Cf. p. 558 *infra*.

(4) *Strains of* "E. coli" *studied*

All the following experiments were made with pure strains of "*E. coli*" isolated and cultivated by the methods just noted. Various strains were obtained in the course of these experiments—not yet recorded—and from other sources: but to avoid repetition, and to give the reader exact data at the outset (for future reference when required), I shall here note a few relevant particulars about the chief cultivated strains of "*E. coli*" used in the present investigations. I give these data very briefly in Table II, which anticipates—in a few instances—findings that will be more fully described a little later.

Table II. *Particulars of the principal strains of* "E. coli" *used in the present experiments*

Strain	Host of origin	Date of isolation	Date when abandoned†	Period of cultivation
λ.	Man (natural infection)	24. xi. 25	19. v. 26 (37)	6 months
NC.	*M. nemestrinus* (natural infection)	3. viii. 27	29. viii. 27 (17)	3 weeks
RC.	*M. rhesus* (natural infection)	19. x. 28	22. iii. 29 (87)	5 months
SC.	*M. sinicus* (natural infection)	3. xi. 28	9. xi. 28 (4)	1 week
DC.	Man (experimentally infected with *RC.*)	13. xi. 28	22. v. 29 (112)	6 months
*MC.**	*H. jacchus* (natural infection)	22. x. 32	—	3¾ years
W.	Ditto	21. i. 33	15. iii. 33 (19)	7½ weeks
MSC.	*M. sinicus* (experimentally infected with *MC.*)	11. iii. 33	2. v. 36 (234)	3¼ years
MRC.	*M. rhesus* (experimentally infected with *MSC.*)	15. ii. 34	12. vi. 34 (73)	4 months
*MSD.**	Man (experimentally infected with *MSC.*)	6. iv. 34	—	2 years
Δ*MC.*	Ditto	18. xii. 34	30. vii. 35 (51)	7½ months
OC.	Man (natural infection)	10. ix. 35	13. xii. 35 (31)	3 months
*UC.**	Man (natural infection)	21. ix. 35	—	8 months

* Strains still under cultivation (May 1936).

† Figures in parentheses denote number of serial subcultures made until strain was given up.

Table II is unavoidably incomplete, so I must add a few explanatory notes. It will be evident that of the 13 strains here mentioned only 10 were of simian origin: the other 3 (Strains λ., *OC.*, *UC.*) were obtained directly from human beings. I never attempted to transmit any of these to monkeys of any species, but used them simply for comparison.

With one exception (Strain *NC.*) all the strains were pure. They contained no other protozoa whatsoever, and were uncontaminated with *Blastocystis*. Their purity was repeatedly checked by inducing them to encyst, and then propagating them from cysts. Both amoeboid stages and cysts of every strain were subjected to careful cytological study, and proved to be morphologically

indistinguishable—at all stages of development—from a typical *E. coli* of Man.[1]

Strain λ. has already been described briefly (Dobell and Laidlaw, 1926*a*). It was one of several similar strains isolated from the same human subject.

Strain *NC.* was obtained *post mortem* from a female *Macacus nemestrinus*.[2] It was a typical "*E. coli*" in every way; but as I could not separate it from the accompanying *E. histolytica*, I used it for no experiments. I record it here because I can find no other record of this amoeba from this species of macaque.

Strain *RC.*, whose origin has already been described,[3] was the only pure strain obtained from *M. rhesus*. But I studied the same amoeba in mixed cultures from the same host (Rosa) on numerous occasions, and kept many impure strains for considerable periods.

Strain *SC.* is mentioned here because it was the only pure strain which I have yet obtained from *M. sinicus*:[4] but I have cultivated and studied dozens of impure strains (mixed with *E. histolytica*) from the same monkey (Susanna), and they all behaved alike.

Strain *DC.* was the most important of several similar strains obtained from the same source. It was abandoned because it ceased to form cysts satisfactorily on continued cultivation (after about 3 months).

Strains *MC.* and *W.* were alike, but were only 2 out of more than 10 isolated from the same marmoset (Willie, *Hapale jacchus*). The former (*MC.*) was originally designated *M.*, and appears under this name in some of the following descriptions. This is because the strain, when originally obtained, was accompanied by a very dense and undesirable bacterial flora, which included *B. pyocyaneus*. As I could not use it for any animal experiments in this state, I cleaned it up by various methods.[5] The final product—Strain *MC.*—was free from all pathogenic bacteria, though the amoebae retained their original cultural properties; and I therefore changed the name of the purified strain (to *MC.*) to distinguish it from the contaminated original (*M.*).

Strain *MSC.* is the only strain which I have yet succeeded in isolating from my monkey Susanna (*M. sinicus*) after infecting her experimentally with Strain *MC.* As she was (and is) also heavily infected with *E. histolytica hominis*, all other attempts—and they are very numerous—have failed. Nevertheless, *MSC.* was undoubtedly pure: and my confidence in its purity was justified by experimental transmission to myself. (The experiment is described in the next section.)

[1] As defined and described in my monograph (Dobell, 1919).

[2] Monkey No. 10 (Dobell, 1931). Strain *Nc.* (*E. histolytica*), and Strain *NT.* (*Trichomonas*: see Dobell, 1934) came from the same source.

[3] See Bishop (1929), and p. 552 *supra*.

[4] Cf. p. 552 *supra*.

[5] This was a very difficult task, which took me a long time. Most of my attempts to remove *B. pyocyaneus*, etc., while preserving the bacteria essential for the development of the amoebae, were failures. I finally succeeded by treating cysts of Strain *M.* with acriflavine—to kill off most of the accompanying bacteria—and then hatching them anaerobically in an atmosphere of CO_2. As several parallel experiments were unsuccessful I cannot recommend this as a general method, and therefore give no further details.

Strain *MRC.* is the only one which I have yet been able to isolate from Rosa (*M. rhesus*, also heavily infected with *E. histolytica*) after infecting her experimentally with *MSC.* It was accompanied by some (unidentified) bacteria which I was never able to eradicate completely, and which made its cultivation extremely troublesome. Owing to these concomitant organisms it was impossible to grow this strain for long in starch-free media, and consequently I could never obtain cysts with any certainty. I was able to prove its purity only by recovering it from cultures containing emetine in high concentration, and then continuing it from the scarce 8-nucleate cysts (typical *E. coli*) formed in a subsequent subculture. This strain was undoubtedly pure—uncontaminated with *E. histolytica*—but very difficult to cultivate and useless for most experimental purposes.

Strains *MSD.* and *ΔMC.* are almost identical strains which I isolated from myself at different dates after my experimental infection with Strain *MSC.* I mention them specially because I used them for various experiments which will be described presently, but they are only two out of many other like strains derived from the same source.

Strains *OC.* and *UC.* here represent 11 different strains which I have cultivated for various periods from 2 human beings. After studying all my simian strains of "*E. coli*" as far as possible, and several others (λ., and similar ones) from Man, it became necessary to examine further human strains for comparison. As it seemed best to obtain material from an entirely new source, I sought the help of my friend Dr Ruth Svensson[1] in Sweden. She kindly mailed me fresh samples of faeces, containing cysts of *E. coli*, from 2 Swedish subjects whom she had herself examined and found to be uninfected with *E. histolytica*; and from these specimens I was able to isolate and cultivate several excellent strains. Those here designated *OC.* were derived from a female patient, with no intestinal disease, in the mental hospital (St Olofs Sjukhus) at Visby: while those called *UC.* came from a normal healthy man residing in Uppsala. Some further details of these Swedish strains of *E. c. hominis* will be recorded later in the present paper.

As already noted, all the foregoing strains of "*E. coli*" were morphologically typical, in every way, at all stages—so far as I have been able to study them. Detailed study of the complete life-cycle—including all the metacystic stages—has as yet been possible, however, only with Strains *MC.* (and its derivatives *MSC.*, *MSD.*, etc.) and *UC.* (and its parallels). Nevertheless, although all the strains were morphologically identical, they were distinguishable and identifiable by the dimensions of their cysts.

It is now well known that *E. coli hominis* consists of several races or strains differing in the sizes of their cysts;[2] and my observations on "*E. coli*" from various monkeys indicate that this is probably true of the species generally. A pure strain can therefore be identified by the dimensions of its cysts; and

[1] I would here gratefully record my indebtedness to Dr Svensson for her generous collaboration.

[2] Cf. Matthews (1919), Dobell (1919, p. 84), etc.

its identity can be established, in different hosts, by the constancy of this character. I have now collected much material which substantiates this statement—not only for *E. coli* but also for *E. histolytica*.

On this point, however, one important qualification is necessary. I have found that, with any pure strain of *E. coli* (or *E. histolytica*), the average dimensions[1] of the cysts are not identical in the host and in the culture-tube: they are always somewhat larger in the latter. That is to say, if any animal is infected with a pure strain of amoebae, then the cysts passed with its faeces have a constant average diameter; while the cysts of the same strain formed in cultures derived from such faeces are likewise constant in size. But the average diameter of the intestinal cysts is not identical with that of the culture-cysts—the latter being always greater. Consequently, if any strain is transferred from one host to another, it can be identified by comparing the cysts formed in the intestines *or* by comparing those in cultures derived from the two hosts. The average dimensions of faecal cysts and cultural cysts—of the very same strain—are different, and they are therefore not exactly comparable.

III. Experiments and Results

The data to be recorded in this section are seemingly incongruous: they include experiments made to determine the viability of cysts, attempts to infect divers animals, observations on the physiological properties of various strains of amoebae, and sundry other matters. Nevertheless, all the experiments and observations are intimately connected—as I shall show later. They are integral parts of a whole, and their bearings on one another will, I hope, soon become evident.

At the outset I must note that the various investigations here described are not now recorded in their historic sequence. The work as a whole has occupied many years, and its several parts have not been done in the order in which they are now presented—this order being adopted merely for convenience of description.

Moreover, this section is essentially a narrative of my own researches, recorded without regard to the results reported by other workers. Their findings—so far as they appear relevant, and are known to me—will be considered in the discussion which follows.[2]

(1) *The Longevity of* "E. coli" *cysts*

It is obviously important to know how long the cysts of *E. coli* can survive outside the body in a condition capable of infecting new hosts; but—as with *E. histolytica*—it is not feasible to determine this point by the use of cysts discharged with the faeces and the experimental infection of a large series of

[1] By "average dimensions" I mean here the mode of a frequency-curve constructed from an adequate series of measurements. The arithmetic mean of such measurements is usually worthless for comparison, owing to the variable occurrence in cultures of outsize (supernucleate) cysts. I hope to discuss this matter in a subsequent instalment.

[2] See p. 580 *infra*.

animals. Nevertheless, it is practicable to determine how long cysts formed in cultures can, under defined conditions, remain viable—that is, able to hatch *in vitro* and give rise to new cultures of normal amoebae once more. I have therefore made numerous experiments by this indirect method.

What I have already written elsewhere[1] about the cultivation of *E. histolytica* from cysts is, *mutatis mutandis*, generally applicable to *E. coli*, so I need not repeat it here: but I would emphasize once more that good experiments can be made with normal and ripe culture-cysts only, and by the use of thoroughly reliable methods of cultivation. Any negative results obtained with poor material, or with unsuitable methods and media, are obviously worthless.

Most of my early experiments—made chiefly in 1925–6 with Strain λ. (from Man)—gave inconclusive results, because I did not then know the best way to keep and hatch cysts of *E. coli*. I merely ascertained that the cysts formed in cultures died rapidly if kept at body-temperature (37°–38° C.) after they had completed their development to the 8-nucleate stage; but that they could survive, and hatch on reincubation (at 37° C.), if kept at the ordinary temperatures of my laboratory (about 15° C., but very variable). The maximum time of survival was about a fortnight.

When I obtained, in 1928, a more suitable strain for study (*RC.*, from *M. rhesus*), I repeated the experiments with somewhat more satisfactory results. For a time I was able to get good crops of culture-cysts, with fair regularity, and these I kept at room-temperature[2] or in the ice-chest (*ca.* 10° C.) and then inoculated into fresh media and incubated at 37° C. The chief results (19 experiments) obtained in this way are summarized in Table III. The

Table III. *Attempts to hatch culture-cysts of* Strain RC.

Age of cysts inoculated	Medium into which cysts were inoculated	Result on incubation*
3 days	Ehs + S	+
3 ,,	HSre + S	+
3 ,,	HSre + S	–
5 ,,	Ehs + S	+
5 ,,	Ehs + S	+
8 ,,	HSre + S	+
10 ,,	Ehs + S	+
13 ,,	HSre + S	+
13 ,,	Ehs + S	–
14 ,,	Ehs + S	–
17 ,,	Ehs + S	–
18 ,,	Ehs + S	+
19 ,,	Ehs + S	–
20 ,,	Ehs + S	–
26 ,,	Ehs + S	–
27 ,,	HSre + S	–
28 ,,	Ehs + S	–
31 ,,	Ehs + S	–
33 ,,	Ehs + S	–

* In this and the following tables the sign + means a culture of normal amoebae was obtained; – means no amoebae found in cultures or subcultures.

[1] Dobell and Laidlaw (1926*a*), Dobell (1927, 1928, 1931).

[2] The weather during most of this time was very cold, and the temperature of my laboratory often below 15° C.

media are designated by the symbols used in a former paper (Dobell and Laidlaw, 1926*a*).

At about this time (1928–9) I was also able to make a few further trials with the cysts of a derivative of Strain *RC.* (Strain *DC.*, "*E. coli*" of *M. rhesus* after passage through Man). But I had great difficulty in obtaining cysts from this strain regularly and copiously, so can record only half-a-dozen satisfactory experiments. These are shown in Table IV.

Table IV. *Attempts to hatch culture-cysts of* Strain DC.

Age of cysts	Medium used	Result
4 days	HSre + S	+
4 ,,	HSre + S	+
8 ,,	HSre + S	+
8 ,,	Ehs + S	–
10 ,,	HSre + S	+
16 ,,	HSre + S	–

The combined results of these 25 experiments (Tables III and IV) indicated that cysts might survive at low temperatures for any time up to 18 days; but they were otherwise inconclusive. Nevertheless, they suggested another conclusion—which later experiments have abundantly confirmed—namely, that the cysts of "*E. coli*" hatch more readily in HSre + S medium than in Ehs + S. For if all the results be taken together—regardless of age and other factors—they show that 7 out of 10 (70 per cent.) of attempts with the former medium were successful, but only 5 out of 15 (33·3 per cent.) with the latter.

Consequently, when I obtained another strain of "*E. coli*" (Strain *M.*, from *Hapale jacchus*) which encysted readily, I repeated the experiments with greater precautions. Ten experiments made with cysts obtained in early subcultures—when the strain was still contaminated with unfavourable bacteria[1]—gave the results shown in Table V. All cysts were kept at room-temperature before incubation.

Table V. *Attempts to hatch culture-cysts of* Strain M.

Age of cysts (days)	Serial sub-culture No.	Medium	Result
4	10	Ehs + S	–
5	4	Ehs + S	–
6	8	Ehs + S	–
7	5	Ehs + S*	–
9	6	Ehs + S	–
9	6	HSre + S	+
9	21	HSre + S	+
10	5	Ehs + S	–
10	7	HSre + S	+
12	18	HSre + S	+

* Culture incubated anaerobically.

These results showed not only that cysts of this strain could live for at least 12 days at room-temperatures, but also confirmed the conclusion that they hatch far more readily in HSre + S medium than in Ehs + S.

The foregoing experiments were made in November 1932, shortly after

[1] Cf. p. 556 *supra*.

I isolated Strain *M*. They showed—in conjunction with many others here unrecorded—that it was useless to attempt to hatch cysts in any medium but HSre+S, but did not indicate the maximum time for which they could survive. In the next few months, therefore, I made another series of similar tests—using the same strain throughout, always incubating the cysts in HSre+S medium, and keeping them at room-temperatures before incubation. The results are summarized in Table VI.

Table VI. *Attempts to hatch culture-cysts* (Strain M.) *by incubation in* HSre+S *medium, after keeping at room-temperature* (15° C. ±)

Age of cysts (days)	Result	Date of experiment[1]
5	+	22. xii. 32
12	+	7. xii. 32
15	+	7. xii. 32
17	+	7. xii. 32
18	+	24. xii. 32
20	+	18. xii. 32
21	+	18. xii. 32
21	+	18. xii. 32
25	+	23. i. 33
26	–	23. i. 33
44	–	7. iii. 33
61	–	7. iii. 33

These experiments proved that cysts of Strain *M*. could live and hatch normally—producing good cultures of typical amoebae once more—after keeping them at room-temperatures for all times up to 25 days. But, as already noted, Strain *M*. was originally accompanied by various undesirable bacteria, which I was later able to eliminate:[2] so I retested the cysts of this strain after purification (*MC*.)—considering such tests, after the removal of harmful influences, likely to be more accurate. A series of careful experiments then gave the results summarized in Table VII.

Table VII. *Attempts to hatch culture-cysts* (Strain MC.) *by incubation in* HSre+S *medium, after keeping at room-temperatures*

Age of cysts (days)	Result	Date of experiment
5	+	11. i. 33
6	+	11. i. 33
25	+	27. ii. 33
30	–	29. iii. 33
31	+	22. iii. 33
35	+	27. ii. 33
44	+	22. iii. 33
45	+	27. ii. 33
51	+	27. ii. 33
56	–	19. iii. 33
57	–	22. iii. 33
64	–	19. iii. 33
64	–	29. iii. 33
70	–	19. iii. 33

[1] In this table, and those next following, I have recorded the date of each experiment because full details are entered under these dates in my note-books, where they can—if necessary—be readily ascertained. The actual experiments were not quite so simple as they here appear, and cover several years.

[2] Cf. p. 556 *supra*.

From this table it will be evident that cysts of "*E. coli*" (Strain *MC.*) are viable at room-temperatures for all[1] times up to 51 days. This was longer than I expected, and suggested that they might live even longer if other factors—especially the temperature—could be more accurately controlled. Accordingly, I made a more extensive series of experiments—continued intermittently for several years—to ascertain the longevity of cysts kept at a uniform low temperature of 10° C. (The temperature was not absolutely constant, owing to the inefficiency of the apparatus at my disposal.) The results of 57 experiments are given in Table VIII.[2]

Table VIII. *Attempts to hatch culture-cysts* (Strain MC.) *by incubation in* HSre+S *medium, after keeping at* 10° C.

Age*	Result	Date	Age	Result	Date	Age	Result	Date
20	+	28. xi. 33	60	+	2. v. 33	100	–	28. v. 34
28	+	6. vii. 33	63	+	6. vi. 33	102	–	11. xii. 33
29	+	12. x. 34	63	+	6. vi. 33	102	+	12. x. 34
30	+	13. xii. 34	66	+	14. viii. 33	103	–	8. vi. 34
31	+	3. iv. 33	67	+	14. viii. 33	104	+	8. vi. 34
31	+	5. viii. 34	70	+	12. v. 33	105	+	8. vi. 34
34	+	30. x. 33	75	+	22. v. 34	109	–	27. ii. 34
37	+	9. iv. 33	79	–	19. xii. 34	110	–	21. x. 34
38	+	1. xi. 33	81	+	29. i. 34	112	–	29. i. 34
38	+	1. xi. 33	83	–	19. xii. 34	114	–	29. i. 34
38	+	1. xi. 33	84	–	19. xii. 34	117	–	29. i. 34
40	+	30. xi. 34	87	+	22. v. 34	120	–	29. i. 34
44	+	16. iv. 33	90	+	25. v. 34	123	–	29. i. 34
48	+	22. xii. 34	95	+	28. v. 34	126	–	29. i. 34
49	+	29. i. 34	95	–	8. vi. 34	129	–	22. v. 34
50	+	27. iv. 34	96	–	13. ix. 33	151	–	16. v. 34
51	+	23. iv. 33	98	–	28. v. 34	152	–	16. v. 34
51	+	26. ii. 35	99	–	8. vi. 34	153	–	16. v. 34
58	+	2. vi. 35	100	–	28. v. 34	155	–	16. v. 34

* The ages of all cysts are in days. No experiments with cysts less than 20 days old are recorded, as they have always been uniformly successful.

This table shows that cysts of "*E. coli*" (Strain *MC.*) regularly survive for 75 days at 10° C., while some may even live for as long as 105 days. After about 11 weeks, however, their survival is uncertain, and no cysts lived for more than 15 weeks.

Whilst making these experiments, I began another series to determine whether cysts could live still longer at still lower temperatures. I kept similar material at a temperature of 1°–2° C., and tested it at intervals in the same way. I now found that cysts treated in this manner survived for all times up to

[1] The failure to hatch cysts kept for 30 days (29. iii. 33) is clearly not significant. Occasional discrepancies of this sort, in experiments involving so many unknown factors, are unavoidable and must therefore be expected. They do not invalidate the general findings.

[2] It should be noted that a positive result—in these tables—does not indicate that *all* or even a majority of the inoculated cysts hatched. In every experiment many cysts were tested simultaneously; and those samples which had been kept for more than a few weeks usually contained numerous cysts which were undoubtedly dead before incubation. After keeping cysts for many weeks, I frequently recovered amoebae in cultures only with difficulty. A positive result, therefore, may mean that no more than one or two cysts—out of perhaps hundreds—survived for the times indicated.

at least 100 days, and often longer. Experiments—up to the time of writing—with cysts aged 100 days or more are given in Table IX.

Table IX. *Attempts to hatch culture-cysts* (Strain MC.), *aged* 100 *or more days, by incubation in* HSre+S *medium, after keeping at* 1°–2° C.

Age (days)	Result	Date
100	+	2. ii. 35
100	–	16. vi. 35
104	+	12. x. 34
110	+	14. ii. 35
113	–	21. x. 34
115	+	20. ii. 35
115	+	20. ii. 35
117	+	2. xii. 35
121	+	24. ii. 36
123	+	24. ii. 36
133	+	13. iii. 36
135	+	23. v. 36
140	–	16. v. 36

I have reported these results in some detail because the data seem to me instructive. They illustrate the importance of various factors in all work of this sort, and demonstrate that conclusions drawn from experiments in which such factors were unknown, or uncontrolled, are worthless. For example, it is impossible to ascertain whether or no cysts are alive (after preservation for various periods) by attempting to hatch them in inappropriate media (cf. Table V). Negative findings may easily be insignificant. Again, it is clear that—even with the best methods of cultivation—the presence of unfavourable bacteria may vitiate the findings (cf. Tables VI and VII). Moreover, when the media and concomitant bacteria are both satisfactory, a further correction is necessary for the temperature at which the cysts have been kept. In general, the lower the temperature (within the limits studied), the longer their life. (Cf. Tables VII, VIII, and IX.)

From all the experiments just recorded it is clear that cysts of *E. coli* (Strain *MC.*) can survive, under otherwise favourable conditions, for 51 days at ordinary room-temperatures: for 105 days at a fairly constant temperature of 10° C.: but for at least 135 days (19 weeks) at 1°–2° C.

Numerous experiments made with other strains of *E. coli* have furnished confirmatory results. Though no strain has yet been tested to the ultimate limit, with control of every factor, I have proved—up to the time of writing—that the cysts of Strain *MSC.* can survive at 2°–10° C. for at least 98 days: those of Strain *MSD.* for 130 days at 2° C.: and those of Strain *UC.* (derived directly from Man) for a minimum period of 86 days at 2°–10° C.

(2) *Infection of Man with* E. coli macacorum

To the best of my knowledge no attempt has hitherto been made to transmit the "*E. coli*" of any macaque to a human being: and I have been able to make only one such experiment, which I shall now describe. It was only possible, of course, after I had obtained a pure strain of this amoeba which

I could cause to encyst *in vitro*. The strain employed was that called *RC.*, from *Macacus rhesus*,[1] and the experimental subject was myself.

I have already recorded several experiments made *in propria persona* with other intestinal protozoa,[2] but they have only an indirect bearing on the present adventure. For this, it is merely necessary to know the evidence for my non-infection with *E. coli* at the date when the experiment was performed (2. xi. 28). It is briefly as follows:

From the winter of 1906–7 until November 1928—a period of approximately 22 years—I examined my stools microscopically, in search of protozoa, more than 250 times. During the years 1925–1928 I also examined them exhaustively by appropriate cultural methods on numerous occasions in the course of various experiments (already partly described) with other amoebae and flagellates. The precise figures, as entered in my note-books from 1906 to November 1928, are 256 microscopical and 60 cultural examinations—all completely negative for *E. coli*. It is therefore highly probable that I was not naturally infected with this amoeba[3] on 2 November 1928.

On this date (2. xi. 28) I swallowed, on an empty stomach, all the cysts from a culture of Strain *RC.* (7th serial subculture, in HSre+S medium). After incubation for 3 days, this culture (made on 28. x. 28) was removed from the incubator and cooled to room-temperature (31. x. 28). The cysts were then washed and concentrated[4] in sterile half-strength Ringer's fluid, and were 2 days old when ingested. Many of them appeared ripe and normal (by microscopical examination of both living and fixed and stained specimens), though they were not very numerous: nevertheless, a rough estimate—made by counting those in a minute measured sample—indicated that the dose taken was at least 350 cysts.

After swallowing these cysts I examined my stools every day—both microscopically and culturally—for 5 weeks, to determine the result of the experiment as exactly as possible. During the whole period I remained physically normal. For 9 days *E. coli* was not discoverable after exhaustive search; but on the 10th day (12. xi. 28) typical amoebae developed in the primary culture, and on the 12th day (14. xi. 28) a few were detected microscopically in the faecal sample. Afterwards they were found intermittently

[1] Cf. p. 552 and Table II, p. 555 *supra*.

[2] See Dobell (1933, 1934, 1935).

[3] I was already infected with *Endolimax nana*, however, in consequence of an earlier experiment (see Dobell, 1933); but this small amoeba is so easily distinguishable from *E. coli* that it caused no difficulties in diagnosis.

[4] The cysts were thoroughly washed in order to free them from excess of bacteria and bacterial products in the medium, so that the ingested material consisted of nothing but cysts, a few dead amoebae, a small number of bacteria, and starch-grains. Most of the bacteria were unknown, but among them was "*Bacillus* I" (Dobell and Laidlaw, 1926*a*, p. 309)—an organism closely resembling *B. dysenteriae* Shiga in its cultural characters and sugar-reactions, though inagglutinable by antiserum for this bacillus. Nothing was known about the pathogenicity of "*Bacillus* I" at this date, though it was derived from a patient suffering from amoebic dysentery. The above experiment, with several others made on other hosts, indicates that it is non-pathogenic when inoculated *per os* in small numbers into healthy animals (man, macaques, and kittens).

in the stools and more regularly in the cultures. The results of all examinations from 2. xi. 28 to 7. xii. 28 (5 weeks) are summarized in Table X.

Table X. *Infection of Man with* E. coli macacorum (*Strain* RC. *from* M. rhesus). *Cysts ingested* 2. xi. 28

	Dates of examinations (1928)											
Nov.	2	3	4	5	6	7	8	9	10	11	12	13
E. coli faeces ...	−	−	−	−	−	−	−	−	−	−	−	−
E. coli cultures ...	−	−	−	−	−	−	−	−	−	−	+	+
Nov.	14	15	16	17	18	19	20	21	22	23	24	25
E. coli faeces ...	+	+	−	−	+	+	−	−	−	−	−	+
E. coli cultures ...	+	+	+	−	+	+	−	−	+	+	+	+
Nov.–Dec.	26	27	28	29	30	1	2	3	4	5	6	7
E. coli faeces ...	+	+	+	+	−	−	−	−	+	+	−	+
E. coli cultures ...	+	+	+	+	−	+	+	+	+	+	+	+

As a result of these findings I was satisfied that I had succeeded in infecting myself experimentally with Strain *RC.*, and so demonstrating that *E. coli macacorum* is transmissible to Man. The amoebae and cysts found in all faecal samples and cultures were morphologically indistinguishable from those in the material inoculated,[1] and differed in no way from typical *E. coli hominis.* I am confident that if any competent protozoologist had examined my stools at this period he would have pronounced me to be infected with an ordinary *E. coli* without any hesitation.

During this period, moreover, I isolated several "pure" strains—free from all other protozoa and *Blastocystis*—from my stools, and cultivated and studied them in detail. The best was Strain *DC.* (isolated 13. xi. 28), with which I made numerous experiments.[2] For a time I was able to obtain cysts from this strain *in vitro*: and their dimensions confirmed their parentage. The average diameter of the cysts of Strain *RC.* (inoculated) agreed closely with that of Strain *DC.* (recovered after infection)—the difference being well within the probable error involved in the measurements.[3]

At the beginning of December (1928) my infection appeared to be well established, so I ceased the daily examinations and examined myself every few days instead—to determine how long the infection would persist. I imagined that it would endure, but in this I was mistaken. On 12. xii. 28 the examination was positive once more, but thereafter—to my surprise—I never succeeded in finding *E. coli* in my stools again. Despite the most exhaustive search, microscopically and culturally, I remained completely negative for this amoeba for the next $5\frac{1}{4}$ years—from 12. xii. 28 to 22. iii. 34, when I attempted to

[1] Numerous measurements of the cysts present in Rosa's faeces previously showed that they had an average diameter of 16·6 μ: while those found in my own, after my experimental infection, had an average diameter of 16·5 μ—thus verifying their origin. Cf. p. 557 *supra.*

[2] Partly recorded elsewhere in the present paper: cf. Table II, p. 555 and Table IV, p. 560. This strain was also studied by Miss Bishop (1929), who tested its resistance to emetine *in vitro.*

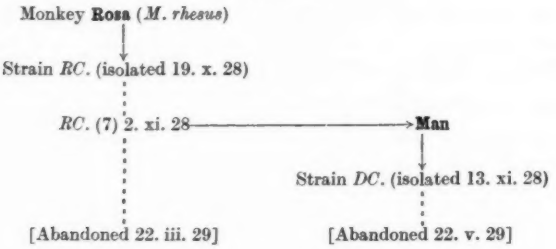
[3] Cf. p. 558 *supra.*

reinfect myself with another strain. During this period I made—on various dates, which are immaterial—altogether 121 negative microscopical examinations and 120 negative attempts to recover the amoebae in cultures.

From all these findings I think it legitimate to conclude that I succeeded in infecting myself with *E. coli macacorum* by ingesting its cysts; but that the infection endured for only 6 weeks (2. xi. 28 to 12. xii. 28), and then died out spontaneously.

The foregoing experiment—with the genealogy of the amoebic strains studied—is exhibited graphically in the accompanying *Scheme* A.

Scheme A


Monkey **Rosa** (*M. rhesus*)
Strain *RC.* (isolated 19. x. 28)
RC. (7) 2. xi. 28
Man
Strain *DC.* (isolated 13. xi. 28)
[Abandoned 22. iii. 29]
[Abandoned 22. v. 29]

(3) *Infection of* Macacus sinicus *with* E. coli jacchi

I have already noted[1] how I was able to obtain an excellent strain of "*E. coli*" from a marmoset (*Hapale jacchus*). As this amoeba (*E. c. jacchi*, Strain *MC.*) appeared to be morphologically and culturally indistinguishable from the species inhabiting Man and the Macaques (*E. c. hominis* and *E. c. macacorum*), I tried to ascertain whether or no it could live also in these two different hosts. The first attempt was made with my tame monkey Susanna, about whom I have already recorded various particulars.[2] Those of present importance are the following:

Susanna (*M. sinicus* = *radiatus* ♀) was naturally infected with "*E. coli*" from the time when she came into my possession (July 1924) until the beginning of 1929—altogether for 4½ years. During this period I studied her infection in detail, and thrice treated her with emetine[3] to eradicate concomitant natural and experimental infections with *E. histolytica*. But these treatments had no effect upon her infection with "*E. coli*", which disappeared spontaneously in the interval between her first and second courses of emetine. The first ended on 12. x. 28, and the second began on 10. iii. 29; and her original infection was certainly present until 27. i. 29—after which date I was never able to demonstrate it. It vanished, therefore, 3½ months after the first and 1½ months before the second emetine-treatment.

[1] Cf. p. 553 *supra*.

[2] Dobell with Bishop (1929), Dobell (1931, 1933, 1934, 1935). Additional data have also been noted already in the present paper.

[3] Dobell with Bishop (1929), Dobell (1931).

I attempted to infect Susanna with *E. coli jacchi* on 11. ii. 33; but as she had previously been infected with "*E. coli*", the evidence for her non-infection at this date is obviously of supreme importance for interpreting the results of this experiment. Briefly it is as follows: From 27. i. 29 (when her natural infection was last demonstrated) until 11. ii. 33—a period of over 4 years—I examined her stools, on various dates, 250 times microscopically and 222 times culturally. Many of these examinations were very thorough—though made, for the most part, in connexion with other experiments already partly recorded—and they were all completely negative for "*E. coli*". To my mind, they furnish almost conclusive evidence that Susanna's natural infection had died out, and that she was therefore uninfected at the date of the present experiment.

I fed Susanna (11. ii. 33) on all the cysts present in 2 rich cultures of Strain *MC.* (serial subculture 62, in Ehs + S medium). The cultures were taken from the incubator 2 days previously (9. ii. 33), the cysts in them washed and concentrated in half-strength Ringer's fluid, and then administered—after keeping at room-temperature—with about 5 c.c. of pasteurized milk.[1] No accurate count of the cysts was made, but some thousands at least were present in the inoculum.

After the feeding I examined this monkey's faeces every day, both microscopically and culturally, for the next 5 weeks (till 17. iii. 33). For 5 days they remained completely negative for *E. coli*: but on the 6th day (17. ii. 33), though the stool was microscopically negative, the culture was positive, and thereafter both were almost invariably so. (There was only a single microscopical negative, with positive culture, on 23. ii. 33.) The experiment is summarized in Table XI.

Table XI. *Infection of* M. sinicus *with* E. coli jacchi (*Strain* MC.). *Cysts ingested* 11. ii. 33

		Dates of examinations (1933)									
	Feb.	11	12	13	14	15	16	17	18	19	20. ii–17. iii
E. coli faeces ...	–	–	–	–	–	–	–	–	+	+	+
E. coli cultures ...	–	–	–	–	–	–	–	+	+	+	+

From these findings I was satisfied that Susanna had acquired an infection with Strain *MC.*—in other words, that I had succeeded in transmitting the "*E. coli*" of a marmoset to a macaque. Susanna's infection, for the last 4 weeks of daily examination, was always easily demonstrable, and she appeared to be heavily and permanently infected. To make sure of this, however, I have continued to examine her faeces—at irregular intervals—up to the time of writing (May 1936); and during the whole of this period of over 3¼ years I have seldom failed to find *E. coli*. Amoebae (and cysts) are still

[1] The drainings from the milk-tube were collected and incubated in suitable medium: and though they were very scanty, they gave an excellent culture of typical *E. coli*—proving that the cysts administered were viable and uninjured.

usually discoverable microscopically in her dejecta—often in large numbers—and develop in cultures with even greater regularity.

After infecting Susanna with Strain *MC.* I made many attempts to recover the amoebae in pure culture from her faeces. But because she was also infected with *E. histolytica* and *E. nana* (and various flagellates and *Blastocystis*) I usually failed. Nevertheless, I succeeded in isolating a pure and freely-encysting strain from faeces passed on 11. iiii. 33—one month after inoculation—and with this strain (*MSC.*) I have been able to make several other experiments.

(4) *Infection of* M. rhesus *with* E. coli jacchi

At the time when I succeeded in infecting Susanna with Strain *MC.*—as just recorded—I made a parallel attempt with another strain of amoebae (*W.*) from the same marmoset, but with a macaque of different species (*M. rhesus*).

The subject of this experiment was **Rosa** (*M. rhesus*), whose previous history was similar to Susanna's.[1] She was originally found to be naturally infected with "*E. coli*", but this infection died out spontaneously. It was demonstrably present from October 1926 until December 1928, and during this time I studied it in great detail. Rosa—like Susanna—was thrice treated with emetine, which had no effect upon her "*E. coli*". By a fortunate coincidence this infection also disappeared spontaneously in the interval between two emetine courses (2 months after cessation of treatment).

Rosa was last found infected with "*E. coli*" (natural infection) on 5. xii. 28. Afterwards she was consistently negative for this species—both microscopically and culturally—until February 1933 when I made the present experiment. Between 5. xii. 28 and 13. ii. 33 (nearly $4\frac{1}{4}$ years) I examined her stools, on various dates, 256 times microscopically and 249 times by means of carefully controlled cultures. All examinations were completely negative for *E. coli*; and in my view stronger evidence of non-infection could hardly be demanded.

On 13. ii. 33 I fed Rosa on all the cysts present in a culture of *E. coli jacchi* (Strain *W.*, serial culture 13, in Ehs+S medium). As in the previous experiment, the numerous cysts were washed and concentrated in half-strength Ringer's fluid, kept for 2 days at room-temperature, and administered with a little milk (with the usual precautions and controls). Afterwards I examined this monkey's faeces every day—microscopically and culturally—for over 6 weeks. During this time the simultaneous experiment with Susanna had succeeded: but to my surprise, Rosa remained negative at every examination. On 1. iii. 33, however, she passed no faeces, so no examinations were possible: while on 7, 11, and 12. iii. 33 the microscopical findings were somewhat doubtful,[2] and on 11. iii. 33 the culture also gave an uncertain

[1] Rosa's history will be found in more detail in earlier instalments (Dobell, 1929 [with Bishop], 1931, 1933, 1934, 1935).

[2] On each of these dates I found an 8-nucleate cyst in the fresh faeces, but decided that they were all probably supernucleate cysts of *E. histolytica* (with which Rosa was also infected at the time), though I was unable to identify them with certainty.

result.[1] With these exceptions, the daily findings were all completely negative for *E. coli* for 46 days following the feeding (13. ii. 33 to 31. iii. 33). I concluded, therefore, that my attempt to infect Rosa had failed.

Meanwhile, I had succeeded in isolating a new strain of "*E. coli*" from Susanna after her experimental infection with Strain *MC*. I therefore decided to try again to infect Rosa with this (*MSC.*, *E. coli jacchi* passed through *M. sinicus*); and accordingly I fed her on all the cysts in a culture of this strain (8th serial subculture in Ehs+S medium). I used the same procedure as before, but the cysts—less numerous, though ripe and normal—were 5 days old, on this occasion, at the time of administration.

Rosa was refed on 31. iii. 33, and I continued the daily examination of her faeces—always both microscopically and culturally—for the next 25 days (till 25. iv. 33). All the examinations were again "negative" for *E. coli*, but towards the end of this period they were most unsatisfactory. For causes of which I am ignorant, the faecal specimens were frequently almost impossible to deal with.[2] Microscopically they showed only a few degenerate amoebae or cysts—impossible to identify with certainty—and often no protozoa of any sort: and to make matters worse, the cultures (usually in HSre+S medium) were frequently worthless. On incubation they became strongly acid, blackened, and very foul, chiefly owing to the presence of amylolytic and proteolytic bacteria: and though *Blastocystis* often developed abundantly, sometimes no protozoa of any sort were discoverable. I was often unable to find even those which I knew this monkey already harboured (*E. histolytica*, *E. nana*, *Enteromonas*): consequently, my failure to find *E. coli* simultaneously was of no diagnostic significance. I found doubtful specimens of this amoeba, it is true, from time to time: but as I could never determine their identity with certainty, or isolate them in cultures, I gave up the daily attempts after 3 weeks of intense but fruitless endeavour.

After 25. iv. 33 I examined Rosa's faeces every few days, in the hope that they would eventually become more amenable to study and so enable me to ascertain the result of my experiment: and on 14. v. 33—after making 5 further inconclusive "negative" examinations—I finally obtained a normal stool which contained a few typical cysts of *E. coli* (and the other protozoa with which the monkey was known to be infected). The culture made on this date also developed normally—giving a good growth of *E. coli* (and other protozoa). Thereafter I had little difficulty in demonstrating the presence of this species in Rosa's faeces, and the infection has persisted up to the time of writing (*i.e.*, for over 3 years).

There can thus be no doubt that I succeeded in infecting Rosa (*M. rhesus*) with *E. coli jacchi*, after its passage through Susanna (*M. sinicus*), but my

[1] On this occasion a few doubtful amoebae were seen in the primary culture. They were probably *E. histolytica*, but I could not determine their species to my satisfaction. *E. histolytica* alone was discoverable in the subculture.

[2] This experience was not unique. I have encountered similar difficulties on several other occasions—especially with the same monkey.

experiment afforded no data beyond this general conclusion. Particulars—such as the length of the "latent" or "incubation" period—were unfortunately indeterminable.

Although I have made many attempts to isolate a pure strain of "*E. coli*" from this monkey's faeces during the last 3 years I have succeeded only once—owing chiefly to her heavy concomitant infection with *E. histolytica*, from which it is usually impossible to separate *E. coli*. A few details regarding the strain successfully cultivated (*MRC.*) have already been noted.[1] Morphologically and culturally it agreed in every way with that inoculated (*MSC.*), but it differed in one notable physiological[2] feature from the strain (*RC.*) which I obtained from the same macaque before this experiment was made. I have no doubt, from all my observations, that Rosa now harbours an *E. coli* (*E. c. jacchi*) which differs from that with which she was originally infected (*E. c. macacorum*).

(5) *Infection of Man with* E. coli jacchi *after passage through* M. sinicus

The experiments described in the preceding sections were instructive in several ways. They convinced me that the "*E. coli*" which I found in my macaques and in a marmoset were morphologically indistinguishable from a typical *E. coli* (of Man): and that the form from the marmoset (*E. c. jacchi*) was able to live in *M. sinicus* and *M. rhesus* enduringly—just like their own "*E. coli*" (*E. c. macacorum*). Moreover, I had ascertained that the "*E. coli*" of *M. rhesus* could inhabit Man; and it thus became necessary, in order to complete the series of experiments, to ascertain whether or no *E. c. jacchi* could likewise live in Man (as a typical *E. c. hominis*). I therefore attempted to infect myself once more—using this time a strain of amoeba derived originally from a marmoset.

I made this experiment in March 1934. As already recorded,[3] my experimental infection with *E. coli macacorum* died out in December 1928: and between that time and the date of the present attempt I examined myself—both microscopically and culturally, with every care and precaution—120 times. As my stools had remained consistently negative for *E. coli* during a period of some $5\frac{1}{4}$ years, I regarded myself as an "uninfected animal" suitable for further experimentation.

For various reasons I decided to make the attempt with Strain *MSC.* (*E. coli jacchi* from *M. sinicus*) rather than with the original strain from the marmoset (*MC.*, from *H. jacchus* direct). The demonstration—if it succeeded—would obviously be more instructive.[4] Accordingly, on 22. iii. 34 I swallowed

[1] Cf. p. 557 *supra*. [2] *Vide* p. 578 *infra*. [3] See p. 565 *supra*.

[4] One of my reasons for selecting Strain *MSC.* rather than *MC.* was to prove that the former was really pure, and that I had—as I believed—certainly succeeded in freeing it from *E. histolytica*. If I had contracted an infection with this species, or gone down with amoebic dysentery after swallowing a culture of *MSC.*, it would have afforded conclusive proof that my methods were defective. On the other hand, the success of the experiment has served to vindicate them completely.

all the cysts in a rich culture of Strain *MSC.* (97th serial subculture, in Ehs + S medium). These cysts had been stored in the ice-chest at a temperature of *ca.* 10° C., and were 5 days old: and I swallowed them on an empty stomach with about 5 c.c. of the half-strength Ringer's fluid in which they had previously been washed and concentrated. I made no accurate count of their number, but certainly ingested at least 1000 ripe normal[1] cysts—together with a few dead amoebae, starch-grains, and divers unknown bacteria present in the culture.

This experiment succeeded almost ideally. I examined my stools every day—both microscopically and culturally—for the next 5 weeks, and found that I was infected with *E. coli* only 5 days after swallowing the cysts. On 27. iii. 34 I made a culture which contained this species on incubation, and for the next 30 days all the cultures were likewise positive. But I could not discover any amoebae or cysts by direct microscopical examination of my stools before 5. iv. 34 (14 days after ingestion); and the microscopical findings were positive and negative with equal frequency afterwards. The daily findings are summarized in Table XII.

Table XII. *Infection of Man with* E. coli jacchi (*Strain* MSC. *from* M. sinicus, *experimentally infected*). *Cysts ingested* 22. iii. 34

		Dates of examinations (1934)											
	Mar.–Apr.	22	23	24	25	26	27	28	29	30	31	1	2
E. coli	faeces ...	–	–	–	–	–	–	–	–	–	–	–	–
	cultures ...	–	–	–	–	–	+	+	+	+	+	+	+
	Apr.	3	4	5	6	7	8	9	10	11	12	13	14
E. coli	faeces ...	–	–	+	+	–	–	–	+	–	+	+	+
	cultures ...	+	+	+	+	+	+	+	+	+	+	+	+
	Apr.	15	16	17	18	19	20	21	22	23	24	25	26
E. coli	faeces ...	–	–	+	+	+	–	–	–	–	–	+	+
	cultures ...	+	+	+	+	+	+	+	+	+	+	+	+

After 26. iv. 34 I continued to examine my stools intermittently in order to ascertain how long the infection would persist. I imagined it might die out—like my previous infection with *E. c. macacorum*—but up to the time of writing (May 1936) it has not done so. It has remained unchanged for over 2 years, and is still easily demonstrable. Since 26. iv. 34 I have made altogether 70 examinations on various dates—always both microscopically and culturally—with the following results: direct microscopical examinations 30 positive, 40 negative; cultures 60 positive, 10 negative. The infection has produced no harmful consequences of any sort: it is, as I anticipated, clinically indistinguishable from an ordinary infection with *E. coli hominis*.

[1] Strain *MSC.* has always produced an unusually high proportion of supernucleate cysts (as many as 25 per cent. in some cultures): and many of these were also present, therefore, on this occasion.

Hitherto I have succeeded in isolating several pure strains of "*E. coli*" from my stools since I reinfected myself, but two—called *MSD.* and Δ*MC.*—have been studied particularly.[1] The first was isolated on 6. iv. 34 (a fortnight after infection) and is still under cultivation at the time of writing: the second was obtained on 18. xii. 34 (9 months after infection), and was voluntarily abandoned about 7 months later. Careful study of these strains has shown them to be morphologically identical at all stages of development, though culturally distinguishable by their concomitant bacteria; while they both agree otherwise, in every essential feature, with their parent strains (*MC.* and *MSC.*) originally derived from *H. jacchus* and *M. sinicus.*

Since it is frequently very difficult to demonstrate an infection with *E. coli*—as already emphasized[2]—and consequently to prove that any experimental animal is really uninfected before use, I have made every possible effort to verify the results just recorded. An obvious criticism is that I *may* have been still infected with *E. c. macacorum* (Strain *RC.*) before I attempted to reinfect myself with *E. c. jacchi* (Strain *MSC.*): and that the amoebae and cysts which are now discoverable in my stools really belong to the first strain—the second having failed to infect. But apart from the improbability that I could have overlooked a persistent infection with the former strain for over 5 years (more than 120 carefully controlled examinations), there is good additional evidence to show that the strain with which I am now infected is different from the one which I acquired in 1928. This evidence rests on an observed physiological difference between these strains—to be described presently[3]—and also upon the dimensions of their cysts. This last piece of evidence appears to me to clinch the matter beyond all reasonable doubt. It is briefly as follows:

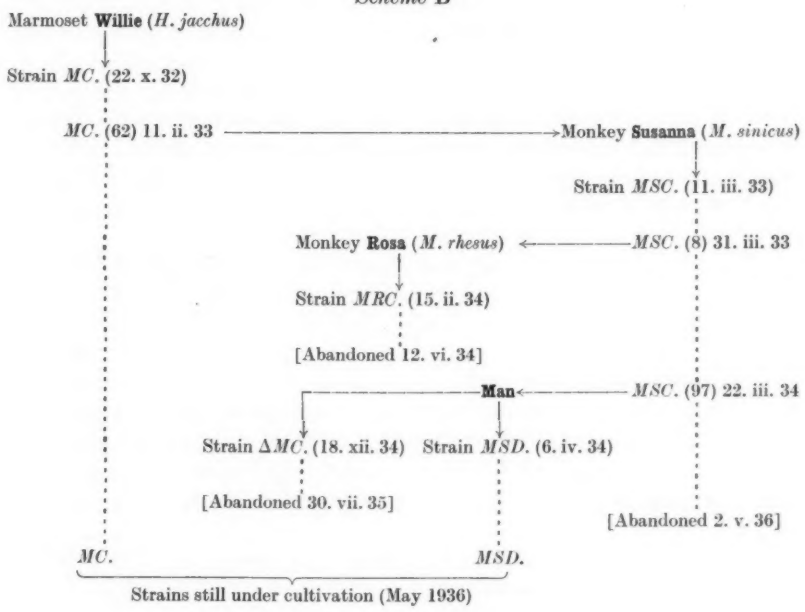
The cysts of Strain *MC.* (*E. coli jacchi*), and all its derivatives, are distinguishable by their larger size from those of Strain *RC.* (*E. c. macacorum*). Numerous measurements of the cysts passed in the faeces of the monkey (Rosa, *M. rhesus*) from which the latter was derived, showed that their average diameter[4] was *ca.* 16·6 μ. When I infected myself with this strain, the cysts in my own stools were found to have an average diameter of 16·5 μ—in good agreement. The cysts in cultures of Strain *RC.* and Strain *DC.* also agreed closely in their dimensions.

I was never able to measure the cysts in the faeces of the host of origin of Strain *MC.* (Willie, *H. jacchus*); but those formed in cultures of this strain correspond in size with those in cultures of all its derivatives (Strains *MSC.*, *MSD.*, etc.). Moreover, the cysts now present in my own stools—after experimental infection with Strain *MSC.*—have an average diameter of *ca.* 20 μ. They can hardly belong, therefore, to the strain (*RC.* from *M. rhesus*, with cysts having a diameter of 16·5 μ) with which I first succeeded in infecting myself, and their true genealogy thus appears to be established—as firmly as *E. coli jacchi* itself in my own body.

[1] Cf. Table II, p. 555 *supra.*
[2] See p. 550 *supra.*
[3] See pp. 577, 578 *infra.*
[4] Cf. p. 565 *supra.*

In order to make clear the sequence of experimental events recorded in this and the previous subsections, and to show at a glance the relations of my variously-named strains of *E. coli jacchi*, I here summarize all results to date in the accompanying *Scheme* B.

Scheme B


Marmoset **Willie** (*H. jacchus*)
Strain *MC.* (22. x. 32)
MC. (62) 11. ii. 33
Monkey **Susanna** (*M. sinicus*)
Strain *MSC.* (11. iii. 33)
Monkey **Rosa** (*M. rhesus*)
MSC. (8) 31. iii. 33
Strain *MRC.* (15. ii. 34)
[Abandoned 12. vi. 34]
Man
MSC. (97) 22. iii. 34
Strain Δ*MC.* (18. xii. 34)
Strain *MSD.* (6. iv. 34)
[Abandoned 30. vii. 35]
[Abandoned 2. v. 36]
MC.
MSD.
Strains still under cultivation (May 1936)

(6) *Duration of Infections*

The foregoing experiments furnish a few data indicating the length of time for which infections with simian strains of *E. coli* may persist in several different hosts. As nothing has been recorded on this subject previously, I may conveniently collect my own experiences at this point.

My data relate to only 3 monkeys[1] (2 macaques of different species and 1 marmoset) and a man. The simian hosts were all found to be naturally infected when they came into my possession, and it is impossible to know how long they had carried their infections formerly. But they all lost their infections spontaneously while under observation, so I know the approximate date of termination for each. I can therefore record the minimal known periods of infection.

[1] The only infection with "*E. coli*" which I have yet seen in *M. nemestrinus* was in an animal (No. 10) which had been caged alone in the Institute for about 15 months before I examined her *post mortem*. It is probable, therefore, that her infection had persisted for at least this period of time; but as no details are available beyond those ascertained at necropsy, I cannot draw any definite conclusions of present importance from this case.

The findings show that one macaque (*M. sinicus*) was naturally infected for at least 4½ years, the other (*M. rhesus*) for rather more than 2 years. The marmoset retained his infection for a little over 1 year.

When the 2 macaques were afterwards inoculated with strains of "*E. coli*" derived from the marmoset, they both acquired infections which have now persisted for over 3 years, and which show, at present, no signs of dying out. The induced infections, therefore, appear to be quite as lasting as the natural.

The one man (myself) experimentally infected with a strain of "*E. coli*" from *M. rhesus* acquired a transient—though otherwise typical—infection, which endured for only about 6 weeks and then vanished. But on reinfection with another strain, from a marmoset, he became more enduringly infected—this strain (*E. c. jacchi*) having already persisted in him for over 2 years. Apparently it is now well established.

As a whole, the experiments show that infections with simian strains of "*E. coli*"—whether natural or experimental—do not always persist for life. Their duration is indefinite. Moreover, an infection with a strain derived from a distantly related host may persist even longer than one from a host more closely related: since, in my own case, a strain of *E. c. jacchi* has produced a more lasting infection than one of *E. c. macacorum*. The duration of an infection may, however, be quite short; for in the only case in which both the beginning and the end could be fairly accurately determined—my own experimental infection with *E. coli macacorum*—the total period was approximately only 6 weeks.

The observations suggest several other conclusions, but these will be discussed—with other results—in the final section.[1] Meanwhile I may summarize the actual data in tabular form (see Table XIII).

Table XIII. *Observed duration of infections with simian strains of* "E. coli"

Host	Duration	Remarks
Susanna (*M. sinicus*)	4½ years (July 1924–Jan. 1929)	Natural infection. Died out spontaneously
,,	3¼ years (Feb. 1933–May 1936)	Experimental infection with *E. c. jacchi*. Monkey still infected
Rosa (*M. rhesus*)	2 years (Nov. 1926–Dec. 1928)	Natural infection. Died out spontaneously
,,	3¼ years (Mar. 1933–May 1936)	Experimental infection with *E. c. jacchi*. Monkey still infected
Willie (*H. jacchus*)	1 year (Oct. 1932–Nov. 1933)	Natural infection. Died out spontaneously
Man (C.D.)	6 weeks (2. xi. 28–12. xii. 28)	Experimental infection with *E. c. macacorum*. Died out spontaneously
,,	2¼ years (Mar. 1934–May 1936)	Experimental infection with *E. c. jacchi*. Man still infected

[1] See p. 580 *infra*.

(7) *Attempts to infect Kittens*

It is now generally allowed that cats cannot be infected with *E. coli hominis*.[1] My own experience agrees with this conclusion, but I have made no extensive study of the subject. Nevertheless, it was necessary—for reasons which will be evident presently—to ascertain whether or no *E. coli jacchi* is able to infect kittens, and produce amoebic dysentery in these animals. I therefore performed several experiments to test this possibility.

Before I record these experiments I may chronicle a couple of attempts previously made with *E. c. hominis*. In 1926 I tried to infect 2 kittens with this amoeba (Strain λ.), because I knew of no similar tests of pure cultivated strains—all previously recorded experiments having been made, apparently, with faecal material. As my attempts were negative, and therefore in agreement with current beliefs, I need note only the following particulars:

Kittens 26 *and* 27—from the same litter, and then about 11 weeks old[2]—were inoculated *per anum* with cultures of Strain λ. (serial subculture 35, in HSre+S medium) on 3. v. 26. Each animal received all the amoebae in a single very rich 2-day culture taken straight from the incubator. Afterwards their faeces were examined whenever possible for the next 3 weeks. (Daily examinations could not be made, as both kittens—though otherwise normal—passed hard formed stools only about twice a week.) As all examinations were completely negative, I finally discharged both animals (on 26. v. 26) as healthy and uninfected to the Farm belonging to the Institute, where they grew into normal cats.

My attempts to infect kittens with *E. c. jacchi* were more elaborate. I used 4 fine healthy animals (Nos. 47, 48, 49, 50, all of the same litter) born in my room at the Institute, from a known normal mother, on 22. x. 34. I reared them with great care, and inoculated them as soon as they were weaned. Each kitten was inoculated twice—with cysts *per os* and with amoebae *per anum*—and 4 different strains of amoebae[3] were used. After inoculation, every stool passed by every kitten[4] was examined both microscopically and culturally till the end of the period of observation.

Every possible care was taken to insure the success of these experiments, and all the materials used were thoroughly controlled. Full records were kept of each animal; but as the results were entirely negative I have recorded only the essential particulars in the accompanying Table XIV. To the information here given, however, the following notes must be added:

[1] Two dissentient opinions expressed recently will be considered later: see p. 584 *infra*.

[2] Both animals had been used previously for other experiments: but as these were unsuccessful, and the faeces of both had remained completely negative for amoebae for 3 weeks before 3. v. 26, I considered them still suitable for the present tests.

[3] The origins of these strains have already been described (see especially Table II, p. 555 *supra*).

[4] Once or twice a small quantity of faeces, passed overnight, was found dried up in the cages when the kittens were first seen in the morning. Such material was never used for cultural study—being unsuitable—and as it was negligible in amount it is here ignored.

Table XIV. *Attempts to infect kittens with various strains of* E. coli jacchi

Kitten No.	Date of inoculation	Strain inoculated*	Mode of inoculation	No. of negative examinations after inoculation	Result
47	(1) 6. xii. 34	*MSD.* (92)	*per anum* (amoebae)	2	Negative
	(2) 15. xii. 34	*MSD.* (96)	*per os* (cysts)	27	No infection. Discharged as normal 15. i. 35
				=29	
48	(1) 7. xii. 34	*MSC.* (149)	*per anum* (amoebae)	2	Negative
	(2) 17. xii. 34	*MSC.* (151)	*per os* (cysts)	26	No infection. Discharged as normal 22. i. 35
				=28	
49	(1) 13. xii. 34	*MC.* (155)	*per os* (cysts)	11	Negative
	(2) 27. xii. 34	*MC.* (207)	*per anum* (amoebae)	11	No infection. Discharged as normal 22. i. 35
				=22	
50	(1) 18. xii. 34	*MSD.* (99)	*per os* (cysts)	16	Negative
	(2) 10. i. 35	Δ*MC.* (14)	*per anum* (amoebae)	10	No infection. Discharged as normal 2. ii. 35
				=26	

* The figures in parentheses denote the serial generations of subcultures inoculated.

Although each kitten was inoculated with amoebae from only one source, I varied the experiments otherwise as much as possible. For example, the cysts administered (various strains) were all of different age. Those (Strain *MSD.*) given to No. 50 (1st expt. 18. xii. 34) were taken straight from the incubator, without cooling: those (Strain *MC.*) given to No. 49 (1st expt. 13. xii. 34) had been kept at 10° C. for 1 month previously. Other cysts used were of intermediate ages.[1] Similarly, no two cultures of amoebae were identical —all being purposely varied in minor details (age, culture-medium, etc.).

The last experiment made (with Kitten 50) differed from the others in that amoebae of 2 different strains were employed. As all the previous attempts had been made with amoebae long cultivated *in vitro* (92nd to 207th cultural generations), and as I had found earlier[2] that *E. histolytica* may lose its infectivity for cats on prolonged cultivation, it seemed desirable to make a final effort with a more recently isolated strain. Accordingly, after I had failed to infect Kitten 50 with cysts of *E. c. jacchi* (Strain *MSD.*, derived from myself), I injected it *per anum* with amoebae of another strain (Δ*MC.*) from the same source—freshly isolated for the purpose some 3 weeks earlier.[3]

The total negative examinations made on each kitten were not very numerous (29, 28, 22, 26, on Kittens 47, 48, 49, 50, respectively), but they were the maximum possible for the period in every case: for all these animals—though otherwise very lively and healthy throughout—passed faeces at infrequent intervals (usually only twice or thrice a week). No kitten suffered from dysentery or any other disorder after inoculation; but to make sure that

[1] The viability of all cysts was controlled, of course, by hatching *in vitro* small samples of the material inoculated.

[2] See Dobell and Laidlaw (1926*a*) and Dobell (1931).

[3] Strain isolated 18. xii. 34. Kitten 50 inoculated with 14th serial subculture 10. i. 35. It is practically impossible to test any cultivated strain sooner, as at least 2–3 weeks are requisite to establish it *in vitro* and demonstrate its purity.

they would all remain healthy, after discharge, I gave them away to friends who have kept them as pets ever since. They all grew into fine normal cats, and with one exception[1] are known to be still alive and vigorous at the time of writing (May 1936).

Although I have been able to make only 8 experiments hitherto, on only 4 kittens, they were sufficiently thorough to satisfy me that *E. coli jacchi* is not readily transmissible to these animals or capable of producing dysentery in them. In this respect, therefore, the "*E. coli*" of *Hapale jacchus*—and its experimental derivatives—appears to differ in no way from a typical *E. coli hominis*.

(8) *Ingestion of Red Blood-Corpuscles by* E. coli

That *E. coli* is distinguishable from *E. histolytica* by its inability to ingest red blood-corpuscles is now almost an axiom of medical zoology.[2] I have shown elsewhere, however, that some strains of *E. histolytica* do not eat red corpuscles—when tested *in vitro*—and that in this species the ability to do so is inconstant: it is a faculty which, in a given strain, may be lost or acquired under certain conditions.[3] I shall now describe a few experiments made to determine the truth about *E. coli*.

As a general routine, I have tested all my pure strains of "*E. coli*", at intervals, by the same methods as I used for *E. histolytica*. The test is extremely simple. I add, aseptically, a drop of freshly drawn blood—usually my own—to a good culture of amoebae, and return it to the incubator immediately: and I then examine it microscopically at intervals to see whether any amoebae have taken up red corpuscles. A positive result is generally obtainable in less than 15 minutes, though it may be necessary to make repeated examinations for several hours to demonstrate a negative.[4]

Strain λ. (*E. coli hominis* isolated from a man studied for many years, and known to harbour this species of amoeba only) was repeatedly tested, and never ingested red corpuscles in any experiment.[5]

Strain *RC*. (*E. c. macacorum* from *M. rhesus*) behaved likewise. No amoeba of this strain, in any test, was ever found to have taken up a single red corpuscle.

Strain *DC*. (*E. c. macacorum* after experimental passage through a man) also behaved in exactly the same way. Whenever I tested it with red corpuscles the results were invariably negative.

The findings from these 3 strains, therefore, were consistent, and conformable with expectation. But when I tested Strain *MC*. (*E. c. jacchi*) in the

[1] Kitten 47—then a lusty tom-cat—left home in October 1935 and has not been heard of since.

[2] Further discussion of this subject, in its traditional aspects, will be found in the next section (p. 586 *infra*).

[3] See Dobell (1931), p. 55 *sqq*.

[4] Cf. Dobell (1931), pp. 56–7.

[5] This strain was offered not only my own red corpuscles but also those of the man from whom it was obtained.

same way, I was astonished to find that it ingested red corpuscles—and occasionally leucocytes also—as readily as any strain of *E. histolytica* which I have ever studied. If a drop of blood be added to any good culture of this strain, most of the amoebae take up red corpuscles almost immediately. Within 10 minutes 75 per cent. (or more) usually contain red cells in their cytoplasm—some having ingested so many (at least 30 to 40) that their number cannot be counted accurately. The appearance of these actively moving amoebae,[1] stuffed with red corpuscles, is exactly like the classical microscopic picture of *E. histolytica*.

Moreover, all my strains of *E. c. jacchi*—derived experimentally from Strain *MC.*, after passage through various hosts—were alike in this respect. Strain *MSC.* (= *MC.* passed through *M. sinicus*) ingested red corpuscles quite as readily as its parent: and so did *MRC.* (= *MSC.* passed through *M. rhesus*) and *MSD.* and Δ*MC.* (= *MSC.* passed through Man).

I must again emphasize the fact that all these strains of *E. coli jacchi* were pure, and uncontaminated with *E. histolytica*. Strain *MC.* itself—the origin of all—was obtained from a marmoset infected with no other species,[2] and it has been subjected to the closest control and scrutiny. It is morphologically, culturally, and developmentally indistinguishable from a typical *E. c. hominis*, and forms characteristic 8-nucleate cysts from which it has been continued *in vitro* over and over again. No cysts resembling those of *E. histolytica* have ever been found in any culture of this strain—thousands of which I have studied in the last 4 years, while I must have examined tens of thousands of cysts. The same is true of Strains *MSC.* and *MSD.* Nevertheless, the reader may have some legitimate doubt about *MSC.* and *MRC.*, since they were both derived from monkeys already known to be infected with *E. histolytica* also. I can only say that I satisfied myself that both these strains were pure—unmixed with *E. histolytica*—by most exhaustive microscopic and cultural control: and that, when I swallowed culture-cysts of Strains *MSC.*, I produced a typical and enduring *E. coli* infection in my own intestine—accompanied by no discoverable *Entamoeba* of any other species, and followed by no signs of amoebic dysentery.[3] All my strains of *E. coli jacchi* were, I repeat, undoubtedly pure. It is inconceivable that any of them was contaminated with *E. histolytica*. This possibility has been excluded absolutely.[4]

[1] They move actively, of course, and ingest red cells readily, only when kept at a constant temperature of 37° C.

[2] I may note here that I attempted to infect this marmoset with *E. histolytica* experimentally (unpublished work), but failed. He was not only uninfected with this species, therefore, but also apparently uninfectible.

[3] Cf. p. 570 *supra*.

[4] Additional evidence of purity may be inferred from their inability to infect kittens, as recorded in the previous subsection (p. 575).—Although it is practically impossible to prove that an animal is uninfected—this being always a question of probability—it is quite feasible to demonstrate that a culture-tube contains only one species of amoeba. Strains *MC.* and *MSD.* are still under cultivation, and are available for examination by anyone who desires to check my observations.

Strain *MC.* (and all its derivatives) was found to ingest not only human red blood-corpuscles (my own) but also those of the marmoset (Willie, *Hapale jacchus*) from which it originated. When I sacrificed him finally, I obtained a sufficient quantity of his blood—by aseptic heart-puncture—to test his own corpuscles with Strains *MC.*, *MSC.*, *MRC.*, and *MSD.* These tests proved that all these strains would ingest Willie's red corpuscles just as readily as my own.

From all the foregoing experiments it would thus appear that *E. coli jacchi* (Strain *MC.*, and its derivatives *MSC.*, *MRC.*, *MSD.*, etc.) differs from a typical *E. c. hominis* (Strain λ.) or *E. c. macacorum* (Strain *RC.* and its human derivative *DC.*) by its power to ingest red corpuscles—like a typical *E. histolytica.* Nevertheless, in every other respect all these various strains of "*E. coli*" were so closely alike that it was obviously necessary to study *E. coli hominis* further before drawing any such conclusion.

Accordingly, I isolated and tested several further strains of *E. coli* from Man. These have already been recorded as *OC.* and *UC.*[1] They were derived from 2 human beings—a man and a woman—resident in Sweden. Both these subjects had been carefully studied by Dr Ruth Svensson, and found by her to be uninfected with *E. histolytica.* From cysts in samples of their faeces[2] I obtained eventually 11 strains of *E. coli*—5 of *OC.* and 6 of *UC.* After proving that these strains were pure, I tested 6 of them (2 *OC.* and 4 *UC.*) with red corpuscles *in vitro*: and, to my surprise, I found that they all ingested red cells as readily and copiously as *E. coli jacchi* or any strain of *E. histolytica* which I have ever yet encountered.

That all these strains of *E. coli hominis* were pure, and uncontaminated with *E. histolytica*, is indisputable. They were all obtained from typical cysts of *E. coli* in faeces of human beings found by previous examination to be uninfected with any other species of *Entamoeba.* Moreover, no strain was tested before I had proved that it was pure by exhaustive cultural and cytological study. Every test was made with amoebae hatched *in vitro* from typical 8-nucleate culture-cysts of *E. coli*, unaccompanied by cysts of any other protozoon: and the tests have been repeated, time and again, with amoebae whose specific purity has been superabundantly demonstrated.

As all *OC.* and *UC.* strains (*E. coli hominis*) gave such dramatically positive results, it was unnecessary—for present purposes—to examine further human strains. The results proved that at least some strains of *E. coli*—naturally inhabiting Man—will ingest red corpuscles as avidly as Strain *MC.* (*E. c. jacchi*) or any strain of *E. histolytica.* Nevertheless, my earlier negative experiments with Strains λ. (*E. c. hominis*) and *RC.* and *DC.* (*E. c. macacorum*) showed equally clearly that other strains of "*E. coli*" exist which do not ingest red

[1] See Table II (p. 555) and p. 557 *supra.*

[2] Microscopical examinations of the original faecal specimens showed that both subjects were also infected with *Endolimax nana*—as already registered by Dr Svensson: but I had no difficulty in eradicating this species from my cultures of *E. coli* by the usual methods.

corpuscles, when tested in a similar manner. I therefore conclude that ability or inability to eat red cells *in vitro* is no constant specific character of *E. coli*.

In my work on *E. histolytica* I found that the power of ingesting red corpuscles, in this species, was also inconstant—even in the same strain. A "positive" strain may become "negative" on prolonged cultivation, while a "negative" strain may become "positive" after passage through a new host: and so on, with disconcerting variations.[1] But to my surprise I have hitherto found no such mutability in *E. coli*. All strains have remained constant throughout. For example, Strain *RC*. (*r.b.c.*−), from *M. rhesus*, was transmitted to Man:[2] and when recovered from this host (Strain *DC*.) was unchanged (still *r.b.c.*−). On the other hand, Strain *MC*. (*r.b.c.*+), from *H. jacchus*, was transmitted to *M. sinicus*, *M. rhesus*, and Man; and all its derivatives recovered and tested from these hosts (Strains *MSC*., *MRC*., *MSD*., Δ*MC*., etc.) were identical (*r.b.c.*+). Even prolonged cultivation *in vitro* has hitherto had no effect on any strain. Today, Strain *MC*.—some 3 years and 8 months after isolation—ingests red corpuscles as eagerly as ever: and the same is true of all its derivative strains, studied for shorter periods.

The constancy of this character in "*E. coli*"—ability or inability of various strains to ingest red corpuscles—has incidentally afforded valuable confirmation of several cross-infection experiments recorded in the present paper. Thus Strain *RC*. was *r.b.c.*− in its original host (*M. rhesus*); and so was *DC*., recovered from the man to whom it was experimentally transmitted. Similarly, *MC*. was *r.b.c.*+ in its host of origin (*H. jacchus*), as were all strains subsequently recovered from animals artificially infected. Strain *MRC*. was *r.b.c.*+ (showing that it was different from *RC*., originally present in the same host), and Strain *MSD*. was also *r.b.c.*+ (showing that it was not identical with *DC*., *r.b.c.*−, with which the host was previously infected experimentally). The constant ability of *E. coli jacchi* (Strain *MC*.) to ingest red cells has, indeed, enabled me to identify—almost to label—all its experimental derivatives in their passage through different animals, and so distinguish them from other strains with which they might otherwise have been confused.

IV. Discussion

Various problems arising from the data recorded in previous sections now require further consideration. Some of these involve matters of fact, others concern questions of interpretation; but all necessitate discussion of relevant results already published by other workers. I shall deal with these problems here as briefly as I can.

The longevity of *E. coli* cysts, outside the body, has previously been studied chiefly by Boeck (1921). He came to the conclusion that cysts (from Man) may remain "viable" in water, at temperatures of 12° to 22° C., for any

[1] See Dobell (1931), pp. 55–60.

[2] Cf. p. 563 *supra*.

time up to 244 days. But his observations were made in the days before cultivation was possible, and he determined whether a given cyst were "alive" or "dead" by its microscopical appearance and the employment of the fallacious "eosin test". There can be no doubt that cultivation affords a far more reliable criterion. If a cyst, on incubation, produces a living amoeba—from which a normal culture can be obtained—then it was indisputably alive: but if a similar cyst, in like circumstances, does not hatch and produce a culture, there is good presumptive evidence that it was dead. Using this more exact method I find that Boeck's estimate (244 days) is nearly twice the true time. The maximum period of survival of *E. coli* cysts (all strains studied) I find to be about 135 days at 1°–2° C., and less at higher temperatures. Even this time, however, is very considerable; and it indicates that cysts of this species, under the most favourable conditions, may remain infective for at least 4 months after they have left their original host.

My own experience leads me, accordingly, to believe that Boeck's figures need drastic correction. On the other hand, I agree entirely with his general conclusion that "The period of infectivity of the human intestinal protozoan cysts is not definitely known and more experiments should be made to determine it".[1]

The duration of infections with *E. coli* has received but scant attention hitherto. It is generally assumed—if not actually stated—that when an infection is once acquired it persists for life: and many of my own records support this belief. I have, indeed, exact data proving that *E. coli* infections in Man may endure unaltered for ten or a dozen years at least. But my experimental results have taught me that transient or short-lived infections may also occur, while seemingly permanent infections may die out spontaneously. I have found that *E. coli macacorum* may live in its natural host for a period of at least 4½ years: nevertheless, in the only two macaques which I have yet been able to study for sufficient time, the infections both disappeared ultimately.

When I succeeded in transmitting *E. coli macacorum* from *Macacus* to Man, the induced infection persisted for only about 6 weeks, and then died out. But I cannot take this as an indication that Man is therefore not a natural host for this amoeba—seeing that infections do not always endure for ever in macaques themselves. The durability of an infection is relative—not absolute. One host may remain infected with a given strain for a long time, another for a short: consequently, the durations of infections afford no criterion for discriminating between strains specifically.

Moreover, there is already good evidence to show that natural *E. coli* infections in Man himself are by no means always permanent. Matthews (1919*a*), who studied a human infection intensively for several years, has published some important findings in this connexion. For a year this infection

[1] Boeck (1921), p. 539. Though I concur in this opinion, I think it could be phrased more felicitously.

was readily demonstrable, by microscopic examination of the stools, and the examinations were then discontinued. But a year later it could no longer be demonstrated, so the case was subjected once more to intensive study. Altogether 80 further examinations were then made in a period of 7 months—on the average, 3 examinations every 8 days. All these were completely negative for *E. coli*. Although no cultures were possible, of course, at the date of these observations, the microscopical evidence alone very strongly indicates that the infection had died out. Matthews drew this conclusion himself—though he did not state it as a certain fact—and shrewdly added "that should *E. coli* again be found in the same case, there will be no means of proving that reinfection has not occurred".

I am personally acquainted with at least one similar case—a man known to have been infected for more than 10 years, but whose infection has now been undemonstrable (microscopically or culturally) for several further years. As yet, however, I have been unable to make sufficient examinations to prove, with absolute certainty, that the infection has been lost.

I would here emphasize the fact that a man or macaque who has spontaneously lost an infection with *E. coli* may none the less be readily reinfected with a different strain. Susanna (*M. sinicus*) and Rosa (*M. rhesus*) and myself all lost our infections with *E. coli macacorum*, after they had endured for various periods; but we were all found to be susceptible to infection with *E. coli jacchi* subsequently. Moreover, all these new infections have now persisted for several years. In my own case, indeed, *E. c. jacchi* has produced a more durable infection (now more than 2 years) than *E. c. macacorum* (6 weeks). This fact is remarkable. I should have expected that an "*E. coli*" from a macaque would infect a man more enduringly and easily than an "*E. coli*" from a marmoset—a host far more distantly related zoologically:[1] but it is obviously impossible to generalize from a single instance.

So far as I am aware nobody has previously tried to infect Man experimentally with "*E. coli*" derived from any monkey. But Knowles and Das Gupta (1934) have recently recorded a possible infection of this sort induced unintentionally. In an attempt to transmit *Balantidium* from a macaque to a man, they fed a volunteer on faecal material from a *Macacus rhesus* containing cysts of *Balantidium*, "*E. coli*", and *E. histolytica*. Unfortunately, no ciliates were afterwards demonstrable in the human subject, who contracted an infection with *E. histolytica* instead. It is added, however, that "on one occasion he has also shown cysts of *Entamoeba coli*—apparently also of monkey origin". The single positive examination for *E. coli* was made during a period of 2 months following the feeding—all other daily examinations being negative: but as only 12 negative examinations were made *before* the experiment, such evidence of transmission from one host to the other is obviously worthless. On the authors' own showing, the man might well have been infected with

[1] It is true I infected myself with *E. coli jacchi* after it had lived for a month in *M. sinicus*: but this does not alter the fact that the strain originated from a marmoset—not a macaque.

E. coli hominis throughout and never have acquired *E. coli macacorum*. It is clearly impossible to draw any definite conclusion from the ambiguous findings reported after this accident.

Although so little evidence has previously been adduced to prove that "*E. coli*" can be transmitted from macaques to men, there is already one published record of success in the opposite direction. Kessel (1928) claims to have proved, in the course of his studies, that "monkeys were experimentally infected with...*E. coli*...of man". This statement must therefore be critically considered here.

The actual data appear to be as follows:[1] Three monkeys altogether were used—No. 5 (*Macacus lasiotis*) and Nos. 13 and 14 (both *M. rhesus*).[2] Monkey No. 5 was "examined three times a week from January 2 to March 8, 1924" and found to be "uninfected" with *E. coli*.[3] Approximately 28 negative examinations were made, therefore, during a period of about 9 weeks. But these examinations were presumably microscopical only—unsupported by evidence from cultures—because *E. coli* had never been cultivated at that date. This is fairly good evidence of non-infection, but it is not conclusive: for I have actual records of more than 28 consecutive negative examinations (microscopical) made on monkeys and men known to be infected with *E. coli*. On an unrecorded date Monkey No. 5 was fed on "5 c.c. of dilute human feces" containing *E. coli*, *E. nana*, *Iodamoeba*, *Giardia*, *Trichomonas*, and *Chilomastix* [whether cysts or free forms is not stated]; and "the routine follow-up examinations were made from three to six weeks after the feeding, when *E. coli*" was "found to have been established".[4] No other evidence of establishment is recorded; and in the light of my own experiences it is clear that these findings are insufficient to prove permanent infection. I can only regard the result of this experiment, therefore, as doubtful. It certainly furnishes no conclusive proof that *E. coli* is transmissible from Man to *M. lasiotis*.

Unfortunately, Kessel's experiments with *M. rhesus* appear to be equally debatable. Monkeys No. 13 and No. 14 were treated with "yatren" [in unspecified dosage] "in an attempt to free them of their intestinal Protozoa".[5] After treatment "each was examined twenty-eight times between January 28 and April 20, 1925", with negative results for *E. coli*. The examinations were, presumably, microscopical only: moreover, No. 13 is entered as negative for *E. coli* even before treatment (Table 4). But as no data are furnished to warrant this entry, and as it is still unproved that yatren is a cure for *E. coli*

[1] I have extracted these data, with some difficulty, from Kessel's final paper (1928). There are several notable discrepancies between this and his earlier record (Kessel, 1924) of the same experiments.

[2] Kessel (1928); Tables 1 (p. 280) and 4 (p. 294).

[3] Kessel (1928), p. 295.

[4] Kessel (1928), p. 295 and Table 4 (p. 294).

[5] Kessel (1928), p. 295. The extracts which follow occur on the same page, but other information is derived from his Tables 1 and 4.

infection of macaques or men, the evidence of non-infection at the date of experiment is obviously inconclusive. In the actual experiments "Both monkeys were fed the intestinal Protozoa indicated in the table, monkey No. 13 being given three feedings on April 22, 23, and 25, respectively, and monkey No. 14 only one feeding on April 21". [Table 4 shows that No. 13 was fed on *E. histolytica*, *E. coli*, *Iodamoeba*, and *Chilomastix*; and No. 14 on *E. histolytica*, *E. coli*, *E. nana*, and *Chilomastix*—no indication being given, in either case, of whether free forms or cysts were present in the faecal material used for these mass-experiments.] "Cysts [no species mentioned] first appeared in the stool of monkey No. 14 on May 2...and on May 11 in the feces of monkey no. 13....Subsequent examination of the feces at intervals until July 6 showed that...*E. coli*...had been established in monkey No. 13 and...monkey no. 14."

In view of my own experience I obviously cannot accept this as evidence of establishment. It seems to me very probable that *E. coli hominis* is transmissible to *M. rhesus*, and Kessel's evidence supports this belief: but his findings—as published—supply no conclusive proof that any species of macaque can acquire permanent infection with any strain of *E. coli* directly derived from Man.

As it may have some bearing upon the present discussion, I must note at this point that Deschiens (1927) has stated that he was able to infect a *Macacus sinicus* by feeding it on "*E. coli*" cysts from a chimpanzee. But the macaque was apparently examined only 8 times microscopically before the experiment was made: and as Mathis (1913) had previously shown—and as I can confirm—this small number of negative examinations is inadequate to prove freedom from infection. The "positive" outcome of this experiment, therefore, should be accepted with considerable reserve.

Few workers, so far as I am aware, have attempted to infect kittens with "*E. coli*" derived from monkeys of any species; and nobody, I believe, has hitherto made such attempts with cultivated pure strains of amoebae. Indeed, the only reference to the subject which I can find in the literature is a casual remark by Brumpt (1909) that the *coli*-like amoebae which he originally discovered in *M. sinicus* "did not show themselves to be pathogenic on inoculation into 4 cats". No details of these experiments appear to have been published.

On the other hand, many workers have tried to infect kittens with *E. coli hominis*, and almost all of them have failed.[1] The inability of this species to infect the cat has, in fact, frequently been invoked as a diagnostic character—distinguishing it clearly from *E. histolytica*. In recent years, however, at least two workers claim to have succeeded in infecting kittens experimentally with *E. coli*, so their findings must now be taken into account.

Brumpt (1926, 1926*a*) inoculated a kitten intrarectally with human faeces containing amoebae identified as *E. coli* and "*E. dispar*"; and a few days later

[1] Cf. Dobell (1919).

he found at necropsy that it still harboured both species. In its caecum superficial ulcers were found: and in or on these the amoebae were discovered. In Brumpt's opinion, these findings show that *E. coli* can parasitize cats—producing in them lesions similar to those characteristic of *E. histolytica*: and on analogy he argues—with the support of various highly questionable records from human cases—that *E. coli* may therefore also be pathogenic to Man. But the findings can obviously be interpreted along more orthodox lines. Most protozoologists—including myself—consider that "*E. dispar*" is a synonym of *E. histolytica*: we do not share Brumpt's belief that *E. histolytica* is really a mixture of two distinct species—"*E. dysenteriae*" (pathogenic to men and cats) and "*E. dispar*" (non-pathogenic). Consequently, I should say—using the customary nomenclature—that in the experiment under discussion Brumpt actually injected into a kitten a mixture of *E. histolytica* (pathogenic to kittens) and *E. coli* (non-pathogenic). The fact that *E. coli* (assuming the identification of this species to be correct) was found later in the amoebic ulcers—produced, as usual, by *E. histolytica*—merely indicates that, on this occasion, *E. coli* survived in the kitten's intestine longer than it generally does. To allege that the normally non-pathogenic *E. coli* made the ulcers, and that the pathogenic *E. histolytica* (arbitrarily called "*E. dispar*", and unjustifiably assumed to be non-pathogenic) afterwards wandered into them, seems merely to invert the facts in order to defend an otherwise untenable taxonomic position. In any case, it is clear that Brumpt's experiment furnishes no evidence to prove that *E. coli*, alone, can parasitize cats; while a method of experimentation which involves the simultaneous inoculation of *E. coli* and *E. histolytica* (even under another name) into kittens, is obviously not one which can be expected to yield conclusive evidence to prove the pathogenicity of the former species.

The other recent worker who claims to have infected a kitten with *E. coli* is Ogawa (1929). He says it is known[1] that cats can be infected with this species, though it is not pathogenic to them. In his own experiment he fed a kitten 3 times on cysts of *E. coli* (from human faeces), and 10 days later found typical amoebae of this species (with which he performed some experiments) in its diarrhoeic stools. Apparently the animal did not acquire amoebic dysentery, but no further details are recorded. It seems necessary to note these findings, though I cannot pretend to understand or interpret them.

It will be evident that Brumpt and Ogawa both believe that *E. coli* can infect the cat. The former believes, however, that this amoeba may be pathogenic to this host, while the latter believes it is not. Brumpt goes even further, and suggests that *E. coli* may also be pathogenic to Man. My own belief—which is shared with most other workers—is that *E. coli* is non-pathogenic to Man (its normal host), and that it cannot usually live in cats at all. All the

[1] His words are "*wie bekannt*": but it is certainly not known to me or, I believe, to most other European and American workers. I have no personal knowledge, however, of Japanese cats—which are possibly peculiar.

experiments—with all strains of this organism—recorded in the present paper support this view. No macaque or marmoset or man spontaneously infected with this species has suffered from dysentery or any other intestinal disorder referable to the infection: no animal experimentally infected with any strain has shown any clinical signs of infection, or suffered any recognizably harmful consequences whatsoever: and no cat which I have attempted to infect with any strain of "*E. coli*" has even become infected.

To relieve the tedium of the present discussion I may mention here a recent paper by Sangiorgi (1933). This writer is aware that attempts to incriminate *E. coli* as a cause of dysentery have hitherto failed: and he also realizes, apparently, that similar efforts to incriminate *Blastocystis*—a harmless vegetable organism present in the intestines of most human beings—have been equally unsuccessful. Consequently, he advances the ingenious hypothesis that, though either organism separately is harmless, the combination of both gives rise to a clinically recognizable form of "mixed dysentery" for which he proposes the fearsome name of "coliamoeboblastocystosis". Although this suggestion scarcely merits attention, I may perhaps add that my present experiments afford no confirmation of it. I myself have been naturally infected with *Blastocystis* for at least 30 years, and my marmoset and all my macaques were likewise infected: yet notwithstanding, all our "*E. coli*" infections—whether natural or experimental—have proved to be innocuous even when combined with this organism. No case of the dreaded coliamoeboblastocystotic dysentery of Sangiorgi has hitherto appeared among my experimental animals.

The last matter of fact which I must here discuss is the ability of *E. coli* to ingest red blood-corpuscles. Theoretically this may be a problem of little interest; yet it is one of very great practical importance, since the diagnosis of amoebic dysentery in a human patient is frequently based on the assumption that *E. coli* does not eat red corpuscles.

Present beliefs on this subject—and they are held almost universally—were well expressed by Wenyon and O'Connor (1917) when they wrote: "Some observers have declared that *E. coli* may, under certain circumstances, phagocyte red blood corpuscles. They have not, however, told us why the entamoebae observed could not have been *E. histolytica*" (p. 68). At the time when this statement was made it appeared unexceptionable. No case of "*E. coli*" containing red corpuscles in its cytoplasm had then been reported which was at all convincing, because concomitant infection with *E. histolytica* had never been rigorously excluded. But Wenyon and O'Connor went further, and laid it down that "If amoebae containing red blood corpuscles are present in a [human] stool, whether evidently dysenteric or not, they are *E. histolytica*" (p. 46): and this statement has since received general assent.

Furthermore, these workers tried to induce *E. coli* to ingest red cells by incubating active amoebae from a fresh stool with a drop of blood; but their experiment was unsuccessful. They saw the corpuscles adhere to the surface

of the amoebae,[1] but never enter them; and O'Connor (1919) afterwards noted—and illustrated—the same phenomenon when *E. coli* was present in the bloody stools of patients suffering from concomitant infections with *Schistosoma*.

A few years later, however, Lynch (1924) repeated the experiment of Wenyon and O'Connor with a different result. He found that when he incubated amoebae in a fresh human stool with a drop of blood, many ingested the red corpuscles readily. Nevertheless, a second experiment, with amoebae obtained by purgation from the same subject, was negative. That the amoebae in question were *E. coli*—unmixed with *E. histolytica*—is attested by Lynch in the following words: "Thorough study of the stools from this individual has failed to reveal the presence of any other protozoon and there is no question of the absence of *Endamoeba histolytica*" (p. 46). But unfortunately the only concrete evidence advanced in support of this statement is a single record of the occurrence of 8-nucleate cysts in a solid stool examined in the interval between his two experiments. I can find no indication, in Lynch's paper, of what he means by "thorough study"—how many times the stools were examined, or for how long a period. Mixed infections are common, and it is far from easy to disprove infection with any species of amoeba. "Non-infection" is, indeed, always a question of probability: by known methods, absence of infection can never be determined absolutely. Consequently, while Lynch's conclusion—that *E. coli* can ingest red cells, like *E. histolytica*—was very likely correct, it can hardly be accepted without more exact data than a personal assurance of his own conviction that he was dealing with a pure infection.

With the discovery of methods of cultivating intestinal amoebae it became possible to study this problem more accurately; and accordingly Drbohlav (1925), when he had succeeded in cultivating *E. coli*, tested this species again *in vitro*. "Numerous attempts to feed the amoebae with red cells of cat or man, either in culture-tubes or on the slide, were made without result."[2] Similar experiments performed at about the same time by Laidlaw and myself[3] were equally unsuccessful; and those which I made afterwards with a strain of "*E. coli*" from *M. rhesus* (and also with its derivative after passage through Man)[4] served only to confirm the general belief.

Up to this point all my findings were consistent, and agreed with current conceptions. All strains of *E. coli* tested appeared to be unable to ingest red corpuscles either *in vivo* or *in vitro*. But the situation was completely changed

[1] Adhesion of red corpuscles to the surface of an amoeba—without ingestion—is very frequently seen in tests *in vitro* such as I have made with *E. coli* and *E. histolytica*. Soon after a drop of blood is added to a culture most of the amoebae appear completely invested with red cells—often completely hidden by them. The phenomenon is particularly noticeable in those strains, of both species, which do not ingest corpuscles, though it also occurs in those which do.

[2] Drbohlav (1925), p. 365—translated.

[3] Dobell and Laidlaw (1926*a*), p. 301.

[4] See p. 577 *supra*.

when I discovered—to my great surprise—that the "*E. coli*" which I isolated from a marmoset would eat red cells just as readily as any strain of *E. histolytica*.[1] My first thought was that the marmoset's "*E. coli*" was possibly not a true *E. coli*, but a closely similar species which could be distinguished by its haematophagous faculty. The more I studied it, however, the more I became convinced that it was distinguishable in no other way. Morphologically, culturally, and in every other respect *E. coli jacchi* appeared to be identical with *E. coli hominis*. The specific identity of these forms was convincingly demonstrated when I finally found[2] that *E. c. jacchi* can live in Man, *M. rhesus*, and *M. sinicus*; and that the infections experimentally established in all these hosts were morphologically indistinguishable from natural infections with *E. c. hominis* and *E. c. macacorum*. Fortunately, moreover, *E. c. jacchi* preserved its haematophagous character in all its transmigrations.

When I succeeded in infecting myself experimentally with a haematophagous strain of "*E. coli*" from *Hapale jacchus*, I was not only satisfied that *E. coli hominis* and *E. c. jacchi* are specifically identical, but I also felt pretty sure that other men—naturally infected elsewhere—must harbour strains of *E. coli* equally capable of ingesting red corpuscles. As already related,[3] I was soon able to verify this inference. The first human strains of *E. coli* which I afterwards isolated and tested *in vitro* were found to ingest red blood-corpuscles just as readily as *E. c. jacchi*—or any strain of *E. histolytica* which I have ever studied.

As all my experiments have been performed with pure cultivated strains of amoebae—proved, by exhaustive control, to be uncontaminated with any other species—I can now state with complete confidence that some strains, at least, of *E. coli* are able and willing to ingest human red blood-corpuscles when they are available. The generally accepted statement that *E. histolytica* ingests red corpuscles, while *E. coli* does not, is certainly false. Both species—though not all strains of either—can and do ingest red cells with equal avidity under suitable conditions. Consequently, ability or inability to eat human red blood-corpuscles affords no criterion for distinguishing between these two species.

It does not necessarily follow that the behaviour of *E. coli* in the test-tube is identical with its behaviour in the bowel. It is conceivable that this species never eats red cells when in the intestine, but only under the peculiar conditions of cultivation.[4] Wenyon (1926), apparently, takes this view: for he says—after noting Lynch's observations—that, in his own experience, ingestion of red corpuscles "never occurs in the intestine, and, if it does, it

[1] See p. 578 *supra*.

[2] See the earlier sections of this paper. The experiments referred to are summarized in *Scheme* B (p. 573).

[3] Cf. p. 579 *supra*.

[4] Analogical evidence for this opinion can be adduced. I have found, for example, that *E. coli* hardly ever eats *Blastocystis* in cultures, though amoebae of this species are frequently seen containing this organism in stools discharged from the human body.

must be such a rare phenomenon that the general rule...that an amoeba with included red cells is almost certainly *E. histolytica*, still holds for all practical purposes" (1, p. 212). But I now have grave doubts of the truth of this statement. When I see the amoebae (undoubtedly *E. coli*) with which I am now infected ingesting red corpuscles with the greatest avidity in cultures, I find it impossible to believe that they are unable to do the same when incubated in my own body. If I were ever to contract non-amoebic dysentery, with blood present in my colon, it seems to me more than likely that amoebae containing red cells would appear in my stools. I should then almost certainly be diagnosed as a case of amoebic dysentery (due to *E. histolytica*)—the dysentery, coupled with amoebae containing red cells, being regarded as convincing proof—and be treated with emetine or some other drug. After treatment, when the dysentery had abated, *E. coli*—typical cysts, and amoebae containing no corpuscles—would alone be found: and it would then be concluded that this was a previously overlooked concomitant infection of no importance. If the drug tried were a new one, a claim for its specific action on *E. histolytica* might even be made with much plausibility.

All this is, of course, pure speculation. The case-history just outlined is hypothetical, though the actual history of my own infection with *E. coli jacchi* is known in detail. I merely visualize what might well happen if it were unknown. Any protozoologist ignorant of my experimental history might easily be deceived by my present *E. coli* infection; and I cannot help thinking that errors such as I foresee may have been made not only in the past, but are even today being made in the diagnosis of "amoebic dysentery".

It has always seemed to me singular that an omnivorous organism like *E. coli* should eschew only red corpuscles. That this species should be so conveniently selective in its tastes seemed almost a divine dispensation to assist us in diagnosis: for even many free-living amoebae are not so fastidious, and—as I noted many years ago[1]—the non-pathogenic *E. ranarum* also eats red cells when they are present in the frog's intestine. But there is no longer any need to marvel, or even to debate this matter. It is now certain that *E. coli* can and does ingest human red blood-corpuscles—under suitable conditions—quite as readily as *E. histolytica* or any other species of amoeba. This is a fact, which must henceforth be taken into serious consideration by all writers of text-books and all clinicians and technicians whose duty it is to diagnose amoebic dysentery. The belief that *E. coli* never ingests red corpuscles is a myth: and in future the finding of "amoebae containing red cells" in a human stool cannot be accepted alone as unquestionable evidence of "amoebic dysentery" or infection with *E. histolytica*.

In conclusion, I may briefly summarize my present evidence in so far as it bears upon the species problem. I have found that a *coli*-like species of *Entamoeba* living naturally in macaques (*E. coli macacorum*, as it is here

[1] Dobell (1909), p. 248.

arbitrarily designated), and another (called *E. c. jacchi*) derived from a marmoset, are both transmissible to Man; while *E. coli jacchi* is also able to infect at least 2 species of *Macacus*. Furthermore, the amoebae in all these hosts appear to differ in no essential feature from one another, or from a typical *E. coli* of man (*E. c. hominis*). Morphologically and culturally all forms are alike, and they also agree in various physiological properties—inability to infect kittens, inability or ability (in certain circumstances) to ingest red corpuscles, the longevity of their cysts, and the duration of their infections in divers hosts. No specific difference has hitherto been discoverable between the "*E. coli*" of Man, of the Macaques, and of a Common Marmoset (*H. jacchus*). There is thus much evidence in favour of the view that the "*E. coli*" living naturally in all these Primates is of one and the same species.

But additional evidence is obtainable from a study of the metacystic development of the organisms here provisionally called *E. coli hominis*, *E. c. macacorum*, and *E. c. jacchi*: and as I propose to describe the metacystic cycle of *E. coli* in the next instalment of this series, I shall reserve further discussion of the species problem for that occasion.

Summary and Conclusions

(i) Amoebae closely resembling *Entamoeba coli* (of Man) have been studied in macaques (*M. rhesus, M. sinicus, M. nemestrinus*) and in a marmoset (*Hapale jacchus*).

(ii) Pure strains of these amoebae have been isolated and cultivated, and carefully compared with similar pure strains of *E. coli* from Man (*E. coli hominis*).

(iii) The complete life-cycles of these various strains—amoebae, cysts, and all stages of precystic and metacystic development—have been studied *in vitro*.

(iv) The "*E. coli*" of macaques (*E. c. macacorum*) was experimentally transmitted to a man, who ingested pure culture-cysts of a strain derived from *M. rhesus*.

(v) The "*E. coli*" of the marmoset (*E. c. jacchi*) was likewise transferred to *M. sinicus*, and from this host again to *M. rhesus* and Man.

(vi) The natural infections observed in *M. sinicus*, *M. rhesus*, and *H. jacchus* all died out spontaneously after persisting for various periods (4½ years, 2 years, and 1 year respectively). The experimental infection with *E. coli macacorum* induced in Man also disappeared spontaneously (after about 6 weeks).

(vii) Attempts to infect kittens experimentally with *E. coli jacchi* were uniformly unsuccessful.

(viii) All infections with "*E. coli*" from every source—whether natural or experimental—have proved to be completely innocuous.

(ix) Culture-cysts of various strains of "*E. coli*" have been found able to live at low temperatures for all times up to about 4½ months (maximum 135 days at 1°–2° C., for a strain from *H. jacchus*).

(x) The "*E. coli*" of *H. jacchus*—and all its experimental derivatives—ingests human red blood-corpuscles *in vitro* as readily as *E. histolytica*.

(xi) Several strains of *E. coli hominis*, isolated directly from naturally infected human beings, have been found to possess the same property.

(xii) Since strains of *E. coli* thus exist—both in men and in monkeys—which eat human red corpuscles avidly, this faculty can no longer be considered as peculiarly characteristic of *E. histolytica*; and the importance of this observation, for the diagnosis of amoebic dysentery, is therefore emphasized. *The statement that* E. histolytica *ingests red corpuscles, while* E. coli *does not, is untrue. It is not possible to distinguish these species by this character alone.*

(xiii) As it has been found impossible to distinguish various strains of "*E. coli*" living in macaques, a marmoset, and men, by any recognizable specific feature—all of them being so closely alike morphologically, physiologically, culturally, and in their ability to inhabit these different hosts—it is concluded that no specific difference actually exists between any of them. The "*E. coli*" of the Macaques and of the Common Marmoset is probably *Entamoeba coli* itself—the species which naturally inhabits Man.

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—— with BISHOP, A. (1929). *Idem.* III. The action of emetine on natural amoebic infections in macaques. *Ibid.* **21**, 446.

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INDEX OF AUTHORS

PAGE

ALVEY, C. H. The Morphology and Development of the Monogenetic Trematode *Sphyranura oligorchis* (Alvey 1933) and the Description of *Sphyranura polyorchis* n.sp. (With Plates X–XI, containing Figs. 1–16) 229

BISHOP, ANN. Further Observations upon a "*Trichomonas*" from Pond Water . 443

BUXTON, P. A. Studies on Populations of Headlice (*Pediculus humanus capitis*: *Anoplura*). I 92

CHEN, H. T. A Study of the Haplorchinae (Looss 1899) Poche 1926 Trematoda: Heterophyidae). (With 12 Figures in the Text) 40

CLEAVE, HARLEY J. VAN. *Tenuisentis*, a new Genus of Acanthocephala, and its Taxonomic Position. (With 4 Figures in the Text) 446

DAS GUPTA, B. M. Observations on the Flagellates of the Genera *Trichomonas* and *Eutrichomastix*. (With Plate VII, containing Figs. 1–15) 195

DAS GUPTA, B. M. *Trichomonas* sp. from the Gut Contents of a Corais Snake. (With 9 Figures in the Text) 202

DAS GUPTA, B. M. On *Eutrichomastix* of some African Snakes. (With 4 Figures in the Text) 206

DAWES, BEN. On a collection of Paramphistomidae from Malaya, with Revision of the Genera *Paramphistomum*, Fischoeder, 1901, and *Gastrothylax* Poirier, 1883. (With 7 Figures in the Text) 330

DOBELL, CLIFFORD. Researches on the Intestinal Protozoa of Monkeys and Man. VIII. An Experimental Study of Some Simian Strains of "*Entamoeba coli*" . 541

DUKE, H. LYNDHURST. Studies of the Effect on *T. gambiense* and *T. rhodesiense* of Prolonged Maintenance in Mammals other than Man; with Special Reference to the Power of these Trypanosomes to Infect Man. I 381

DUKE, H. LYNDHURST. Studies of the Effect on *T. gambiense* and *T. rhodesiense* of Prolonged Maintenance in Mammals other than Man; with Special Reference to the Power of these Trypanosomes to Infect Man. II. 391

ELTON, CHARLES and KEAY, GLADYS. The Seasonal Occurrence of Harvest Mites (*Trombicula autumnalis* Shaw) on Voles and Mice near Oxford. (With 1 Figure in the Text) 110

EVANS, A. C. Studies on the Influence of the Environment on the Sheep Blow-Fly *Lucilia sericata* Meig. IV. The Indirect Effect of Temperature and Humidity acting through certain Competing Species of Blow-Flies. (With 2 Figures in the Text) 431

FAUST, ERNEST CARROLL and CHUNG-CHANG TANG. Notes on New Aspidogastrid Species, with a Consideration of the Phylogeny of the Group. (With 14 Figures in the Text) 487

FOWELL, R. RALPH. Observations on the Sporozoa Inhabiting the Gut of the Polychaete Worm *Polydora flava* Claparède. (With 22 Figures in the Text) . . . 414

PAGE

GIEMAN, QUENTIN M. and RATCLIFFE, HERBERT, L. Morphology and Life-cycle of an Amoeba Producing Amoebiasis in Reptiles. (With Plates VIII and IX, containing Figs. 1–39 and Fig. 1 in the Text) 208

HERTIG, MARSHALL. The Rickettsia, *Wolbachia pipientis* (gen. et sp.n.) and Associated Inclusions of the Mosquito, *Culex pipiens*. (With Plates XIII–XVII, containing Figs. 1–41, and 1 Figure in the Text) 453

HOARE, CECIL A. Morphological and Taxonomic Studies on Mammalian Trypanosomes. I. The Method of Reproduction in its Bearing upon Classification, with special reference to the *Lewisi* Group. (With 1 Figure in the Text) . . . 98

IYENGAR, M. O. T. Entry of *Filaria* Larvae into the Body Cavity of the Mosquito. (With Plate VI and 3 Text-Figures) 190

JOBLING, B. A Revision of the Subfamilies of the Streblidae and the Genera of the Subfamily Streblinae (Diptera Acalypterae) including a Redescription of *Metelasmus pseudopterus* Coquillett and a Description of Two New Species from Africa. (With 5 Figures in the Text) 355

LAWRENCE, R. F. The Prostigmatic Mites of South African Lizards. (With 23 Figures in the Text) 1

LEESON, H. S. Further Experiments upon the Longevity of *Xenopsylla cheopis* Roths. (Siphonaptera). (With 3 Figures in the Text) 403

LEIGH-SHARPE, W. HAROLD. New Parasitic Copepoda from Naples. (With 9 Figures in the Text) 63

LEIGH-SHARPE, W. HAROLD. Some Rare and New Parasitic Copepoda, etc., from Brighton and Elsewhere. (With 2 Figures in the Text) 410

LUCKER, JOHN T. Preparasitic Moults in *Nippostrongylus muris*, with Remarks on the Structure of the Cuticula of Trichostrongyles. (With 13 Figures in the Text) . 161

LYSAGHT, AVERIL M. A Note on the Adult Female of *Anguillulina aptini* (Sharga). A Nematode Parasitising *Aptinothrips rufus* Gmelin. (With 1 Figure in the Text) 290

LYSAGHT, AVERIL M. A Note on an Unidentified Fungus in the Body Cavity of two Thysanopterous Insects. (With 1 Figure in the Text) 293

MACLEOD, JOHN. *Ixodes ricinus* in Relation to its Physical Environment. IV. An Analysis of the Ecological Complexes Controlling Distribution and Activities. (With 5 Figures in the Text) 295

MACLEOD, JOHN. Studies in Tick-borne Fever of Sheep. II. Experiments on Transmission and Distribution of the Disease 320

METTAM, R. W. M and CARMICHAEL, J. Turning Sickness, a Protozoan Encephalitis of Cattle in Uganda. Its Relationship with East Coast Fever. (With Plate XII) 254

PATTEN, RUTH. Notes on a New Protozoon, *Piridium sociabile* n.gen., n.sp., from the Foot of *Buccinum undatum*. (With 15 Figures in the Text) 502

PINKERTON, HENRY. Criteria for the Accurate Classification of the Rickettsial Diseases (Rickettsioses) and of their Etiological Agents. (With Plates IV and V, containing Figs. 1–10) 172

ROTHSCHILD, MIRIAM. The Process of Encystment of a Cercaria Parasitic in *Lymnaea tenera euphratica*. (With 8 Figures in the Text) 56

PAGE

SIMON, JAMES R. and SIMON, FELIX. *Philonema agubernaculum* sp.nov. (Dracunculidae), a Nematode from the Body Cavity of Fishes. (With 5 Figures in the Text) 440

TATE, P and VINCENT, M. The Biology of Autogenous and Anautogenous Races of *Culex pipiens* L. (Diptera: Culcidae). (With 5 Figures in the Text) . . . 115

THEILER, HANS and FARBER, SEYMOUR M. *Trichomonas muris*, Parasitic in the Oxyurid Nematodes, *Aspicularis tetraptera* and *Syphacia obvelata*, from White Mice. (With 1 Figure in the Text) 149

THORPE, W. H. On a New Type of Respiratory Interrelation between an Insect (Chalcid) Parasite and its Host (Coccidae). (With 24 Figures in the Text). 517

WARWICK, TOM. The Parasites of the Muskrat (*Ondatra zibethica* L.) in the British Isles 395

WIGGLESWORTH, V. B. Symbiotic Bacteria in a Blood-sucking Insect, *Rhodnius Prolixus* Stål. (Hemiptera, Triatomidae). (With 4 Figures in the Text) . . 284

WOOLCOCK, VIOLET. *Chloromyxum pristiophori*, a New Species of Myxosporidia Parasitic in the Gall-bladder of *Pristiophorus cirratus* (Saw-Shark). (With Plates I and II, containing Figs. 1–19) 72

WOOLCOCK, VIOLET. Monogenetic Trematodes from some Australian Fishes. (With Plate III and 4 Text-Figures) 79

YAKIMOFF, W. L. and MATIKASCHWILI, I. L. Coccidiosis of the Grey and Stone Partridge 146

INDEX OF SUBJECTS

PAGE

Agamidae, mites parasitic on South African 20

ARACHNIDA

. Acarina
. Prostigmata
. *Geckobia* Mégnin
. *australis* Hirst 4
. *hemidactyli* n.sp. 14
. *hewitti* n.sp. 8
. *homopholis* n.sp. 15
. *karrooica* n.sp. 18
. *namaquensis* n.sp. 10
. *natalensis* n.sp. 17
. *oedurae* n.sp. 6
. *ovambica* n.sp. 4
. *pachydactyli* n.sp. 12
. *phyllodactyli* n.sp. 15
. *rhoptropi* n.sp. 11
. *tasmani* n.sp. 6
. *transvaalensis* n.sp. 17
. *Pterygosoma* Peters
. *aculeatum* n.sp. 25
. *agamae* Peters 25
. *armatum* n.sp. 30
. *bedfordi* n.sp. 24
. *hirsti* n.sp. 22
. *longipalpe* n.sp. 25
. *melanum* Hirst 21
. *transvaalense* n.sp. 28
. *triangulare* n.sp. 28
. *Trombicula autumnalis* Shaw, on voles and mice 111
. Ixodoidea
. *Ixodes ricinus* in relation to its physical environment 295

Buccinum undatum, notes on a new protozoon, *Piridium sociabile*, n.gen., n.sp. from the foot of 502

Coccidiosis of the grey and stone partridge 146

CRUSTACEA

. Copepoda
. Copepoda, new parasitic, from Brighton and elsewhere 410
. Copepoda, new parasitic, from Naples 63
. *Alella berecynthia* n.sp. 65
. *Caligus rabidus* n.sp. 410
. *Hatschekia ischnon* n.sp. 63
. *getto* n.sp. 63

PAGE

CRUSTACEA

Ismardis spartacus n.sp. 68
Lernaeocera lusci (Bassett-Smith) 410
Nemesis robusta van Beneden 410
Parategastes haphe n.sp. 67
Pycnogonum littorale (Stroem) 410
Filaria larvae, entry of, into the body cavity of mosquito 190
Fungus, note on an unidentified, in the body cavity of two Thysanopterous insects . 293
Geckonidae, mites parasitic on South African 2
Harvest mites (*Trombicula autumnalis* Shaw), the seasonal occurrence of, on voles and mice near Oxford 110
Headlice (*Pediculus capitis*: Anoplura), studies on populations of 92

INSECTA

ANOPLURA
Pediculus humanus capitis, studies on populations of 92
APHANIPTERA
Xenopsylla cheopis Roths, further experiments upon the longevity of . . 403
DIPTERA
Culex fatigans Wied., entry of *Filaria* larvae into 190
pipiens L., biology of autogenous and anautogenous races of . . . 115
pipiens L., rickettsia of 453
Lucilia sericata Meig., influence of environment on 431
Mansonia (*Mansonioides*) *annulifera* Th., entry of *Filaria* larvae into . . 190
Metelasmus pseudopterus Coquillet (1907), redescription of 370
Nycteribosca bequaerti sp.n., description of 374
Raymondia intermedia sp.n., description of 377
HEMIPTERA
Rhodnius prolixus Stål., symbiotic bacteria in 284
Saissetia (*Lecanium*) *hemisphaerica* (Targioni), respiratory relationship with Chalcid 517
HYMENOPTERA
Encyrtus infelix (Embleton), respiratory relationship of larva of, with host . 517
THYSANOPTERA
Aptinothrips rufus Gmelin, nematode parasitising 290
Lizards, the prostigmatic mites of South African 1
Mites, prostigmatic, of South African lizards 1
Muskrat (*Ondatra zibethica* L.), parasites of, in the British Isles 395
Pristiophorus cirratus (saw-shark), a new species of Myxosporidia parasitic in gall-bladder of 72

PROTOZOA

AMOEBAE
morphology and life-cycle of, producing amoebiasis in reptiles . . 208
Entamoeba coli, an experimental study of some simian strains of . . . 541
invadens Rodhain 1934, morphology and life-cycle of . . . 209
COCCIDIA
Eimeria kofoidi sp.n., in partridges 147
Polyrhabdina polydorae Caul. & Mesn. (?), in gut of polychaete worm . . 427
Selenidium axiferens sp.n. 423

PAGE

PROTOZOA

. FLAGELLATA
. *Eutrichomastix* of some African snakes 206
. spp., observations on 195
. *Trichomonas keilini* from pond water, further observations upon . . . 443
. *muris*, parasitic in oxyurid nematodes from white mice . . 149
. sp. from the gut contents of a Corais snake 202
. spp., observations on 195
. *Trypanosoma brucei*, infectivity of 393
. *gambiense* and *rhodesiense*, effect on, of prolonged maintenance in
. mammals other than man 381, 391
. Trypanosomes, morphological and taxonomic studies on mammalian . . 98
. MYXOSPORIDIA
. *Chloromyxum pristiophori*, in gall-bladder of saw-shark 72
. SCHIZOGREGARINES
. *Piridium sociabile*, n.gen. et n.sp., from the foot of *Buccinum undatum* . . 502
Respiratory interrelation, a new type of, between an insect (Chalcid) parasite and its host (Coccidae) 517

RICKETTSIAE

. Rickettsia, *Wolbachia pipientis* (gen. et sp.n.) and associated inclusions of the
. mosquito, *Culex pipiens* 453
. Rickettsial diseases (Rickettsioses), criteria for the accurate classification of, and
. of their etiological agents 172
Sheep blow-fly, *Lucilia sericata* Meig., studies on the influence of the environment on, 431
Streblidae, revision of the subfamilies of 355
Symbiotic bacteria in a blood-sucking insect, *Rhodnius prolixus* Stål. (Hemiptera, Triatomidae) 284
Tick-borne fever of sheep 320
Tenuisentis, see Vermes, Acanthocephala 446
Turning sickness, a protozoan encephalitis of cattle in Uganda. Its relationship with East Coast Fever 254

VERMES

. ACANTHOCEPHALA
. *Tenuisentis*, a new genus and its taxonomic position 446
. NEMATODA
. *Anguillulina aptini* (Sharga), a nematode parasitising *Aptinothrips rufus*
. Gmelin 290
. *Aspicularis tetraptera* from white mice, *Trichomonas muris* parasitic in . . 149
. *Filaria malayi* Brug., in mosquitoes 190
. *Nippostrongylus muris*, preparasitic moults in, with remarks on the
. structure of the cuticula of Trichostrongyles 161
. *Philonema agubernaculum* sp. nov. (Dracunculidae), a nematode from the body
. cavity of fishes 440
. *Syphacia obvelata*, from white mice, *Trichomonas muris* parasitic in . . 149
. *Wuchereria bancrofti* Cobb., in mosquitoes 190
. POLYCHAETA
. *Polydora flava* Claparede, sporozoa in gut of 414

PAGE

VERMES

TREMATODA

Aspidogastrids, notes on new species of 487
Calicotyle inermis n.sp. 82
Cercaria parasitic in *Lymnaea tenera euphratica*, process of encystment of . 56
Cotylaspis sinensis n.sp. 487
Gastrothylax elongatus Poirier, 1883 333
cobboldii Poirier, 1883 335
crumenifer (Creplin, 1847) 336
spatiosus Brandes, 1898 337
synethes Fischoeder, 1901 339
Haplorchinae (Looss 1899) Poche 1926 (Trematoda: Heterophyidae), a study of the 40
Haplochoris milvi (Gohar 1934) 54
pumilio (Looss 1896) 43
taichui (Nishigori 1924) 47
yokogawai (Katsuta 1932) 50
Lophotaspis orientalis n.sp. 490
Megalocotyle helicoleni n.sp. 80
Microcotyle sillaginae n.sp. 84
bassensis (Murray) 87
victoriae n.sp. 88
Monogenetic Trematodes from some Australian fishes 79
Paramphistomidae from Malaya 330
Paramphistomum cervi (Schrank, 1790) 341
explanatum (Creplin, 1849) 341
siamense. (Stiles and Goldberger, 1910) 346
gotoi Fukui, 1926 348
cotylophorum Fischoeder, 1901 350
orthocoelium Fischoeder, 1901 352
Sphyranura oligorchis Alvey 1933, morphology and development of . . . 229
polyorchis n.sp., description of 229
Stichocotyle cristata n.sp. 492

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CONTENTS

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PAGE

HERTIG, MARSHALL. The Rickettsia, *Wolbachia pipientis* (gen. et sp.n.) and Associated Inclusions of the Mosquito, *Culex pipiens*. (With Plates XIII–XVII, containing Figs. 1–41, and 1 Figure in the Text) 453

FAUST, ERNEST CARROLL and CHUNG-CHANG TANG. Notes on New Aspidogastrid Species, with a Consideration of the Phylogeny of the Group. (With 14 Figures in the Text) 487

PATTEN, RUTH. Notes on a New Protozoon, *Piridium sociabile* n.gen., n.sp., from the Foot of *Buccinum undatum*. (With 15 Figures in the Text) . 502

THORPE, W. H. On a New Type of Respiratory Interrelation between an Insect (Chalcid) Parasite and its Host (Coccidae). (With 24 Figures in the Text) 517

DOBELL, CLIFFORD. Researches on the Intestinal Protozoa of Monkeys and Man. VIII. An Experimental Study of Some Simian Strains of "*Entamoeba coli*" 541

INDEX OF AUTHORS 595

INDEX OF SUBJECTS 599

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